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The vital role for nitric oxide in intraocular pressure homeostasis

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Abstract

Catalyzed by endothelial nitric oxide (NO) synthase (eNOS) activity, NO is a gaseous signaling molecule maintaining endothelial and cardiovascular homeostasis. Principally, NO regulates the contractility of vascular smooth muscle cells and permeability of endothelial cells in response to either biochemical or biomechanical cues. In the conventional outflow pathway of the eye, the smooth muscle-like trabecular meshwork (TM) cells and Schlemm's canal (SC) endothelium control aqueous humor outflow resistance, and therefore intraocular pressure (IOP). The mechanisms by which outflow resistance is regulated are complicated, but NO appears to be a key player as enhancement or inhibition of NO signaling dramatically affects outflow function; and polymorphisms in NOS3, the gene that encodes eNOS modifies the relation between various environmental exposures and glaucoma. Based upon a comprehensive review of past foundational studies, we present a model whereby NO controls a feedback signaling loop in the conventional outflow pathway that is sensitive to changes in IOP and its oscillations. Thus, upon IOP elevation, the outflow pathway tissues distend, and the SC lumen narrows resulting in increased SC endothelial shear stress and stretch. In response, SC cells upregulate the production of NO, relaxing neighboring TM cells and increasing permeability of SC's inner wall. These IOP-dependent changes in the outflow pathway tissues reduce the resistance to aqueous humor drainage and lower IOP, which, in turn, diminishes the biomechanical signaling on SC. Similar to cardiovascular pathogenesis, dysregulation of the eNOS/NO system leads to dysfunctional outflow regulation and ocular hypertension, eventually resulting in primary open-angle glaucoma.

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Keywords

Conventional outflow; Glaucoma; Ocular hypertension; Schlemm's canal; Shear stress; Trabecular meshwork

1. Introduction

Despite the vast production of scientific work involving nitric oxide (NO) (more than 176,000 entries in PubMed to date), the discovery of NO as a molecule with physiological relevance is a relatively recent event (Moncada and Higgs, 2006; Murad, 2004). NO was previously known as a pollutant, but it was not until the late 1970's that the role of NO as a signaling molecule started to become clear. Furchgott and Zawadzki (1980) described the existence of a molecule that was produced by endothelial cells and relaxed smooth muscle. At that point, they called the "mediator" endothelium-derived relaxing factor (EDRF). Early work on EDRF revealed that acetylcholine, bradykinin and histamine all stimulated its production, and that EDRF acted via soluble guanylyl cyclase and was inhibited by hemoglobin and methylene blue (Furchgott et al., 1984; Ignarro et al., 1986). Some years earlier, Murad and colleagues were already working on the activity of guanylyl cyclase in response to NO (Arnold et al., 1977), independently of the EDRF studies.

NO meets all criteria for a prototypical gasotransmitter (Mustafa et al., 2009): it is light weight (MW = 30D); it is highly permeable with respect to lipid bilayers; it can be endogenously generated; it requires no exclusive cognate cell surface receptors (but it has several critical intracellular macromolecular targets); it has associated derivatives (e.g.: superoxide, nitrite, nitrate, nitrous oxide, peroxynitrite) that are critical to its function; and most importantly, it serves as a signaling molecule for a wide variety of essential physiologic functions including, as we will argue, intraocular pressure (IOP) regulation (Wang, 2018). There are other gasotransmitters, including carbon monoxide (Bucolo and Drago, 2011) and hydrogen sulfide (Han et al., 2019), but these will not be covered in this review because their role in ophthalmic physiology has not been studied as extensively as NO.

Leading to the identification of EDRF as NO, there were studies describing the short half-life of the molecule (Gryglewski et al., 1986) and the realization that EDRF was a free radical, due the observation that most of its inhibitors had redox properties that lead to the generation of superoxide (O_2^-) (Moncada et al., 1986). It was in 1987 when Ignarro et al., 1987a, 1987b and Palmer et al. (1987) confirmed that EDRF was NO. Later, Palmer, Moncada and colleagues showed that L-arginine served as a substrate for NO production (Palmer et al., 1988), by NO synthase (NOS) (Moncada et al., 1989), which was isolated in 1990 (Bredt and Snyder, 1990). After 1990, the scientific production involving NO increased exponentially until it plateaued around the year 2000; since then, more than 7000 papers on NO are published every year. Due to the significance of these discoveries, NO was named "molecule of the year" in 1992 by *Science* (Culotta and Koshland, 1992) and Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad won the Nobel Prize in physiology or medicine in 1998 'for their discoveries concerning NO as a signaling molecule in the cardiovascular

system' (Nicholls, 2019). Since then, NO has been shown to be a key mediator in most vascular beds in the body, including those in the eye.

In the first part of this review, we provide an overview of NO biology and its role in endothelial function and dysfunction. This will provide the necessary background to discuss the role of NO in aqueous humor dynamics, IOP regulation and pathology leading to primary open-angle glaucoma (POAG), which accounts for 75% of glaucoma globally and over 50% of glaucoma-related blindness (Quigley and Broman, 2006). Importantly, lowering IOP is the only efficacious means to slow POAG progression. Over the past decade, it has become clear that NO is a key regulator of IOP homeostasis within the conventional outflow pathway. In the second part of the review we provide a comprehensive examination of pioneering work in this realm, which together supports a novel mechanism by which NO modulates conventional outflow resistance to maintain IOP. Proper functioning of the conventional outflow pathway maintains IOP within a few mmHg throughout the lifetime of most people (Gabelt and Kaufman, 2005; Toris et al., 1999). However, its dysfunction is responsible for high IOP in POAG (Grant, 1951), which has motivated industry partners to develop technologies that target NO signaling to treat POAG.

2. Nitric oxide/nitric oxide synthase basics

NO is produced by NOS, which are a family of enzymes made of three isozymes transcribed from three different genes: neuronal NOS (nNOS, *NOS1*), inducible NOS (iNOS, *NOS2*), and endothelial NOS (eNOS, *NOS3*). nNOS and eNOS are constitutively expressed, while iNOS is "inducible", being produced under pathological conditions as a mediator of the immune response. nNOS and eNOS are found in different cell types and tissues, but their names come from the cell types in which they were discovered and where they are more abundant (Bredt and Snyder, 1990; Förstermann et al., 1991; Goureau et al., 1993). Due to its importance in IOP homeostasis, endothelial-derived NO from eNOS will be the primary focus of this review.

2.1. NO production and eNOS structure

eNOS is an enzyme of 1203 amino acids with a molecular weight of 133 kDa that is translated from the gene *NOS3* found in position 7q35–7q36 in the human genome. eNOS catalyzes the oxidation of the amino acid L-arginine to produce NO, L-citrulline and water (Palmer et al., 1988). The reaction is facilitated by an electron flux generated from the conversion of NADPH to NADP (Fig. 1).

The structure of eNOS supports the chemical reactions that lead to the production of NO and its regulation (Fig. 1). The carboxyl terminal (C_t) contains a reductase domain, where NADPH is converted into $NADP^+ + H^+$ with the consequential release of electrons. Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are co-factors that facilitate the transport of these electrons towards the oxygenase domain in the amino terminal (N_t). To further promote electron transport, calcium (Ca^{2+}) activated Calmodulin (CaM) binds to the CaM binding domain located between the reductase and oxygenase domains. CaM coupling is essential for efficient electron transfer and, therefore, enzyme function. In the oxygenase

domain, a heme group and the essential cofactor tetrahydrobiopterin (BH_4) facilitates the production of NO (Chen et al., 1996; Sheta et al., 1994).

eNOS requires the formation of a homodimer to be active. The monomers mostly interact at the oxygenase domain, where the binding site for BH_4 and the heme moiety form an active pocket (Crane et al., 1998; Fischmann et al., 1999). Additionally, there are 2 cysteine residues (one per monomer) that form either a disulphide bridge or a zinc thiolate cluster (Raman et al., 1998). Finally, a N_t hook domain stabilizes the two monomers when the dimer is being formed (Crane et al., 1999). BH_4 is also necessary for the formation of a stable dimer. Otherwise, the electrons are transferred into the oxygen molecule and superoxide is produced instead of NO (Xia et al., 1998), a process known as eNOS uncoupling (Vásquez-Vivar et al., 1998). The structure of the N_t containing fatty acylation by myristic and palmitic acid facilitates binding to lipid bilayers such as the Golgi apparatus and caveolae (Garcia and Sessa, 2019).

2.2. eNOS modulators

eNOS requires both the interplay with accessory molecules as well as posttranslational modification of its amino acids in order to synthetize NO. Requisite activities for NO formation include: dimerization of the monomers facilitated by BH_4, replacement of caveolin-1 (CAV1) for Ca^{2+} dependent CaM, association with heat shock protein 90 (hsp90), phosphorylation of Ser1177 and dephosphorylation of Thr495. A brief description of eNOS modulators and modifications are given below and displayed schematically in Fig. 2:

CaM is the main protein interacting with eNOS (Bredt and Snyder, 1990). Divalent calcium ion is require for CaM to stably bind to the CaM binding domain of eNOS. Without CaM, there is no effective electron transfer from FMN in the reductase domain to the heme group in the oxygenase domain (Daff et al., 1999; Nishida and Ortiz de Montellano, 1999). Typically, 200–400 nM Ca^{2+} are necessary for CaM to bind to eNOS (Förstermann and Sessa, 2012).

CAV1 is an inhibitor of eNOS function. Importantly, CAV1 is a protein scaffold interlaced in the phospholipid bilayer that is essential to the biosynthesis of caveolae, and loss of CAV1 results in loss of caveolae (Drab et al., 2001). Caveolae are specialized cellular domains that form “cup-shaped” invaginations in cellular membranes (Richter et al., 2008; Schlörmann et al., 2010), and are implicated in a variety of physiological processes including mechanosensing (discussed below), regulation of eNOS signaling and (García-Cardeña et al., 1996, 1997; Patel et al., 2008), and mechanotransduction (Albinsson et al., 2008; Joshi et al., 2012; Yu et al., 2006). eNOS is sequestered in caveolae (Shaul et al., 1996), with CAV1 binding to the CaM binding domain of eNOS, and negatively regulating it (Bucci et al., 2000; García-Cardeña et al., 1996, 1997; Ju et al., 1997; Michel et al., 1997): Therefore, binding of CaM to the CaM binding domain disrupts the interaction with CAV1, which activates eNOS (Michel et al., 1997). Moreover, CAV1 can also play a role in protein trafficking, which can limit the accessibility of eNOS to signaling cues preventing further downstream activity (Wang et al., 2009).

Hsp90 is a protein that enhances eNOS function through multiple mechanisms. Hsp90 promotes the affinity of eNOS for CaM (Pritchard et al., 2001), and it is necessary for Akt to interact with eNOS (García-Cardeña et al., 1998; Takahashi and Mendelsohn, 2003a). This interaction leads to eNOS phosphorylation (Fontana et al., 2002). Hsp90 also protects Akt from degradation (Wei and Xia, 2005). Additionally, Hsp90 can activate eNOS independently of Ca^{2+} (Takahashi and Mendelsohn, 2003b), contributing to eNOS activation in response to hypoxia (Chen and Meyrick, 2004). Finally, NO negatively regulates Hsp90 by S-nitrosylation as a means to limit further NO production (Martínez-Ruiz et al., 2005).

Post translational modifications determine the cellular location of eNOS, which in turn define the type of signals (biochemical or biomechanical) that regulate its exposure and activity. For example, palmitoylation and myristoylation of its N_t glycine promotes localization in caveolae (Shaul et al., 1996).

Phosphorylation of certain serine and threonine residues have a different impact on eNOS activity. For instance, phosphorylation of Ser1177, Ser635 and Ser617 stimulate eNOS function, mostly by promoting CaM binding (Bhandari et al., 2006) and preventing CaM-eNOS dissociation (Ser1177) (Dimmeler et al., 1999; McCabe et al., 2000). In contrast, phosphorylation of Thr495 and Ser116 inhibit eNOS function (Fleming et al., 2001; Greif et al., 2002; Kou et al., 2002).

S-nitrosylation in response to NO itself inhibits eNOS activity, and de-nitrosylation is necessary for the enzyme to function. Nitrosylation appears to affect eNOS by modifying the substrate or co-factor binding sites, thereby inhibiting electron transport at the interface between monomers and by promoting disassembly of the homodimer (Erwin et al., 2005; Ravi et al., 2004).

2.3. eNOS activation and regulation

The activity of eNOS is complex and exquisitely regulated, involving both spatial and temporal controls along with the synergy of multiple factors. Initially, eNOS requires a cellular location that allows its access to extracellular signaling cues to activate the enzyme. In particular, eNOS needs to be located at the cell membrane. At thi At this location, signals take the form of biomechanical (i.e. shear stress) or biochemical downstream signals from signal transduction (i.e. from G protein coupled receptors, GPCR). The activation of eNOS occurs through 2 main mechanisms, (*i*) mobilization of intracellular Ca^{2+} and (*ii*) phosphorylation in response to PI3K/Akt cascades. Additionally, the interaction with the co-factors described above is also necessary. Finally, negative feedback mechanisms are in place in order to inhibit NO production when needed - these are mostly NO itself and eNOS internalization (Fig. 2).

The actin cytoskeleton plays an important role in eNOS translocation from intracellular compartments to the cell membrane (Govers and Rabelink, 2001). Additionally, eNOS is also internalized and recycled. For this reason, the balance between G-actin and F-actin regulates eNOS activity, with greater G-actin being associated with more eNOS activity (Kirsch et al., 2013; Su et al., 2003). Regarding these processes, two accessory proteins have been found: eNOS interacting protein and eNOS trafficking inducer protein (Dedio et al.,

2001; Icking et al., 2005) (Fig. 2). These proteins are involved in eNOS internalization and trafficking and both inhibit eNOS function (Dedio et al., 2001; Zimmermann et al., 2002). eNOS is less active when not attached to caveolae, due to the lack of signaling when the protein is internalized and the limited access to Ca^{2+} (Jagnandan et al., 2005). However, eNOS becomes hyperactive in the absence of caveolae in CAV1 knock out (KO) mice, as CAV1 negatively regulates eNOS activity (Drab et al., 2001; Elliott et al., 2016). Since the biochemical content of plasma membrane does not change, eNOS myristoylation and membrane localization is likely to still occur in CAV1 KO mice, allowing the interaction of eNOS with Ca^{2+} and other signaling molecules.

In the caveolae, eNOS is accessible to extracellular signaling (biomechanical and biochemical) that would promote eNOS activity by either mobilizing Ca^{2+} into the cell or by phosphorylating its serines. For the relevance of biomechanical signals in outflow function, we will discuss in detail how those influence eNOS activity in the next section. In terms of biochemical signals that can activate eNOS, the number and nature of factors are very broad and are mostly mediated through GPCRs (Fig. 2). Thus, mobilization of intracellular Ca^{2+} can be triggered by molecules such as bradykinin, acetylcholine, histamine, adenosine and thrombin, which interact with their cognate receptors. In contrast, serine phosphorylation can be triggered by guanine nucleotide exchange factors and guanosine 5′-triphosphate (GTP) activated proteins, insulin and hormones such as estrogen and platelet derived mediators (Dudzinski et al., 2006). Interestingly, estrogen as an activator of eNOS may be particularly important as considerable evidence suggests that relative estrogen deficiency in the reproductive and post-reproductive years contributes to elevated IOP (Vajaranant et al., 2016) and POAG (Vajaranant and Pasquale, 2012). Molecules like vascular endothelial growth factor (VEGF) and sphingosine-1-phosphate can activate eNOS through both mechanisms, Ca^{2+} internalization and phosphorylation (Jagnandan et al., 2005). VEGF deserves special attention, as it is the most potent eNOS stimulator. VEGF can promote Ca^{2+} mobilization (He et al., 1999), phosphorylate eNOS at Ser1177 within 5 min, de-phosphorylate Ser116 within 30 min (Kou et al., 2002), and induce eNOS de-nitrosylation (Erwin et al., 2005). Therefore, VEGF may activate eNOS though multiple regulation points that act over a range of different time scales.

Finally, eNOS also has negative regulatory feedbacks to avoid NO overproduction. These mechanisms include internalization and trafficking, and Hsp90 and eNOS S-nitrosylation (Erwin et al., 2005; Martínez-Ruiz et al., 2005; Ravi et al., 2004).

2.4. Biomechanical activation of eNOS

Biomechanical activation of eNOS occurs in response to hydrodynamic shear stress or mechanical stretch. Several molecular pathways and initial mechanotransducers are involved in the biomechanical activation of eNOS, discussed below and summarized in Fig. 3. In general, these pathways converge to increase intracellular Ca^{2+} or to activate PI3K/Akt. Biomechanical activation of eNOS has been a topic of other reviews (Balligand et al., 2009; Davies, 1995). Here, we focus on the main points necessary to understand the mechanosensitive role of eNOS in the context of aqueous humor outflow.

Local biomechanical regulation of eNOS via shear stress is a principal factor controlling arterial diameter. Shear stress is a crucial hemodynamic force in the vasculature as it stimulates changes in gene expression, release of vasoactive substances, changes in cell metabolism and morphology (Davies, 1995). In endothelial cells, acute shear stress causes a transient rise in intracellular Ca^{2+}, which activates eNOS and NO production via Ca^{2+}/CaM signaling, however, sustained shear stress triggers NO release in a Ca^{2+}/CaM-independent manner (Kuchan and Frangos, 1994). Sustained shear-induced NO production requires eNOS phosphorylation by kinases such as protein kinase A (Balligand et al., 2009; Fleming, 2010) or protein kinase B (Dimmeler et al., 1999; Fulton et al., 1999).

CAV1 and caveolae act as mechanotransducers for shear stress in endothelial cells, initiating and integrating signaling cascades (such as eNOS) in response to shear. In fact, Yu et al. (2006) showed that loss of endothelial CAV1 and caveolae expression resulted in reduced shear stress-induced physiological responses in blood vessels such as vessel dilation/constriction and eNOS activity, and this loss of function was rescued when CAV1 was reintroduced. Caveolae appear to act as scaffolds that hold together signaling molecules associated with specific pathways (such as eNOS) in an inactive state, and upon mechano-stimulation with shear stress, they facilitate fast signal transduction to downstream effectors allowing endothelial cells to respond rapidly and efficiently (Frank and Lisanti, 2006; Frank et al., 2003; Lisanti et al., 1994). Consistent with this notion, caveolae are enriched with molecules involved in shear-induced eNOS activation. For example, cationic arginine transporter-1 and several molecules that regulate intracellular Ca^{2+} concentration localize in caveolae (Isshiki and Anderson, 1999; Isshiki et al., 2002; McDonald et al., 1997). Shear-induced eNOS activation can occur via β1 integrins on the apical surface of endothelial cells, and this process is dependent on caveolae structural integrity (Yang et al., 2013). In addition, shear stress-induced integrin activation occurs downstream from the VEGF receptor (VEGFR)/vascular endothelial cadherin (VE-cadherin)/platelet endothelial cell adhesion molecule-1 (PECAM-1) mechanosensory complex (Tzima et al., 2005), and a component of this complex, VEGFR-2, associates with CAV1 (Labrecque et al., 2003). Caveolae have also been shown to “shelter” shear-sensitive signaling molecules such as VEGFR-2 from shear stress, and to allow exposure of these molecules when the plasma membrane becomes stretched (Shin et al., 2019), suggesting caveolae play an important role in regulating the intensity of response to shear. Finally, caveolae are also necessary for glycocalyx-mediated mechanotransduction and subsequent downstream eNOS signaling (Zeng et al., 2014).

Another important mechanosensor is Piezo-type mechanosensitive ion channel component 1 (PIEZO1). PIEZO1 is a non-specific mechanically-gated cation channel expressed on the plasma membrane, linking mechanical signals to immediate biological functions (Coste et al., 2012; Ge et al., 2015). PIEZO1 is activated by shear stress and regulates shear stress-induced elevation of intracellular Ca^{2+}, cell alignment, vascular tone, blood pressure regulation, adenosine triphosphate (ATP) release, and NO production (Li et al., 2014; Wang et al., 2016; Wong et al., 2018), processes necessary for blood vessel formation during development, hemodynamics, and vascular structure during adulthood (Li et al., 2014; Ranade et al., 2014). Wang et al. (2016) showed that knockdown of PIEZO1 inhibited shear-induced increases in intracellular Ca^{2+}, eNOS activity, and ATP release. Potentiation of

these parameters, including phosphorylation of Akt, was observed following administration of a PIEZO1 activator (Yoda1), mimicking the effects of shear stress on endothelial cells (Wang et al., 2016). Moreover, eNOS protein was reduced and VEGF-dependent phosphorylation of eNOS at Ser1177 was abolished in human umbilical vein endothelial cells depleted of PIEZO1 and aorta from mice with haploinsufficiency of PIEZO1 (total KO of PIEZO1 is embryonically lethal) (Li et al., 2014).

In endothelial cells, a dominant mechanosensory complex involved in shear stress-induced integrin activation is comprised of platelet endothelial cell adhesion molecule-1, VE-cadherin, and VEGFR-2 complex (Tzima et al., 2005). VEGFR-3 has also been identified as a component of the complex (Coon et al., 2015). Shear force is transmitted from the apical membranes of endothelial cells through the cytoskeleton to cell-cell and cell-extracellular matrix adhesion points. Thus, integrins and adherens junctions sense mechanical changes in tension and act as mechanotransducers in endothelial cells (Davies, 1997). Shear stress causes integrin activation, mediating physiological responses such as cell alignment, gene expression, and modulation of signaling pathways (Tzima et al., 2001, 2002, 2003).

The mechanotransduction activity of VE-cadherin, VEGFR-2 and PECAM-1 are intimately intertwined. Both PECAM-1 and VE-cadherin are major cell-cell adhesion molecules in endothelial cells (including Schlemm's canal) with a cytoplasmic domains that binds to β- and γ-catenins (Albelda et al., 1990; Newman and Newman, 2003; Vest-weber, 2008). VE-cadherin is a major component of the adherens junction complex (Taddei et al., 2008) and essential for controlling vascular permeability at associated tight junctions (Vincent et al., 2004). VEGFR-2 is a receptor tyrosine kinase that binds to and mediates angiogenic effects of VEGF (Rahimi, 2006). Together, this mechanosensory complex is necessary for the activation of a subset of shear-dependent signaling pathways. Cell lines deficient in PECAM-1 or VE-cadherin do not exhibit cell alignment or integrin activation in response to shear stress, and re-expression of these receptors rescues shear-induced integrin activation and cell alignment (Tzima et al., 2005). Separate application of mechanical strain on PECAM-1 and VE-cadherin with magnetic beads bound to receptor-specific antibodies demonstrated direct mechanotransduction by PECAM-1 and not VE-cadherin, suggesting that PECAM-1 is the direct transducer of shear stress in this mechanosensing complex (Collins et al., 2012; Tzima et al., 2005), although $P2Y_2$ receptor and G_q/G_{11} might be required for the upstream mechanical activation of the VEGFR/VE-cadherin/PECAM-1 complex (Wang et al., 2015b). Onset of shear stress induces PECAM-1 phosphorylation, which triggers ERK (Osawa et al., 2002), and Src activation (Tzima et al., 2005). Shear stress also induces the VE-cadherin-dependent association of PECAM-1 and β-catenin with the p85 subunit of phosphoinositide 3-kinase (PI(3)K) (Tzima et al., 2005). VE-cadherin and its binding partner, β-catenin, act as a unified adapter protein in this complex, enabling the physical association of PECAM-1 and VEGFR-2. VE-cadherin is required for the ligand-independent activation of VEGFR-2 by Src following induction of flow or mechanical strain on PECAM-1 (Conway and Schwartz, 2012; Tzima et al., 2005). The interaction between VE-cadherin and VEGFR-2 was originally thought to be indirect via β-catenin (Lampugnani et al., 2006), however more recent evidence suggests a direct interaction of transmembrane binding domains within both proteins (Coon et al., 2015). Shear-induced VEGFR-2 activation is required for phosphorylation of the p85 subunit of PI(3)K, which occurs within

15 s after the onset of shear stress, and continues to increase for several minutes (Tzima et al., 2005). Therefore, PECAM-1 is required for the mechanosensation of shear stress and Src activation, while VE-cadherin enables the transmission of this signal to PI(3)K. PI(3)K goes on to activate integrins and mediate other signaling pathways such as Akt and eNOS (Hughes and Pfaff, 1998; Tzima et al., 2005). Dysregulation of the VEGFR/VE-cadherin/PECAM-1 complex leads to endothelial dysfunction. For example, PECAM-1 KO mice exhibit loss of shear stress-induced NO-dependent dilation of vessels (Bagi et al., 2005; Fleming et al., 2005; McCormick et al., 2011). Moreover, shear-induced PECAM-1 phosphorylation activates Src, and Src mediates eNOS expression in acute and chronic responses to shear stress (Davis et al., 2001). Furthermore, inhibition of β1 integrin activation reduced shear-induced signaling involving Src-family kinases, PI (3)K, Akt and eNOS (Yang and Rizzo, 2013).

The Tie2/PI(3)K/Akt signaling pathway is another mechanosensory pathway involved in shear-induced NO production in human endothelial cells. Akt is one of the main regulators of shear stress-induced eNOS phosphorylation, and is activated downstream of shear-induced PI(3)K phosphorylation (Dimmeler et al., 1999; Fulton et al., 1999; Lee and Koh, 2003). Akt directly phosphorylates eNOS in a Ca^{2+}-independent manner, causing the production of NO (Dimmeler et al., 1999; Fulton et al., 1999). Tyrosine kinase with immunoglobulin and epidermal growth factor homology domain-2 (Tie2) is widely expressed in endothelial cells and plays a role in vasculogenesis, angiogenesis, and hematopoiesis during development (Sato et al., 1995). Shear stress activates Tie2 receptor tyrosine kinase in human endothelial cells, with maximum phosphorylation occurring at 5 min (Lee and Koh, 2003). Lee and Koh (2003) suggested that Tie2 is the main contributor to the PI(3) K/Akt signaling pathway due to the extent, amount, and time window of Tie2 activation relative to shear-induced PI(3)K/Akt activity. This theory was later supported when Yang et al. (2012) discovered that Tie2 knockdown inhibited shear-induced activation of Akt and eNOS in human early endothelial progenitor cells. Therefore, the Tie2/PI(3) K/Akt signaling pathway is an important regulator of shear-induced eNOS activity and NO production in endothelial cells.

The glycocalyx plays an important role in mechanotransduction and shear-induced NO production in vascular endothelium (Pahakis et al., 2007; Tarbell and Ebong, 2008; Tarbell and Pahakis, 2006; Weinbaum et al., 2003, 2007; Yao et al., 2007). The luminal surface of most vascular endothelium is coated with a variety of membrane-bound macromolecules that establish the hydrophilic glycocalyx layer, including glycoproteins with terminal sialic acids, proteoglycans, and glycosaminoglycans (Pries et al., 2000; Tarbell et al., 2014). The GAGs that make up part of the glycocalyx are heparan sulfate, chondroitin sulfate, and hyaluronic acid (Pahakis et al., 2007). Transmembrane syndecans constitute some of the major protein core families on the endothelial cell plasma membrane with GAG attachments sites, and the cytoplasmic tails of the syndecans associate with the cytoskeleton, enabling the transduction of force from the lumen throughout the cell (Pahakis et al., 2007). Enzymatic degradation of components of the glycocalyx such as heparin sulfate, hyaluronic acid, and sialic acid, but not chondroitin sulfate, inhibits shear-induced NO production in bovine aortic endothelial cells (Florian et al., 2003; Mochizuki et al., 2003; Pahakis et al., 2007).

2.5. NO signaling

As a gasotransmitter, NO is a lipophilic, diatomic and uncharged free radical that despite its short half-life can act as both autocrine and paracrine signaling molecule due to its ability to diffuse between cell membranes without a transport system (Gryglewski et al., 1986; Hakim et al., 1996; Kelm, 1999). NO has a very short life, especially due to the high reactivity it has with superoxide anion and oxygen, and its high affinity for the heme moiety of hemoglobin; thus, its bioavailability is reduced in environments rich in these products, as such, the half-life of NO in blood has been reported to range between 0.05 and 1 s (Hakim et al., 1996; Jia et al., 1996; Kelm, 1999; Straub et al., 2012; Villamor et al., 2003). In contrast, NO's half-life is significantly longer in biological fluids with no heme content but containing serum albumin, such as aqueous humor in the outflow pathway, which have significant implications in terms of NO signaling and IOP homeostasis. At saturating concentrations (2 mM) the half-life of NO in water is < 1 s, and it proportionally increases when the concentration of NO is reduced, for instance, the half-life of NO at 5 nM is 70 h (Beckman and Koppenol, 1996). To our knowledge, the half-life of NO in aqueous humor has not been reported.

There are two main signaling mechanisms and molecular modifications that NO exerts on target cells: *i*) through activation of soluble guanylyl cyclase (sGC) and *ii*) through post-translational modification of proteins. NO has a high affinity for the heme group in sGC. Upon binding, NO disrupts a His-Fe(II) bond, altering its configuration to an active state (Derbyshire and Marletta, 2012). Active sGC catalyzes the conversion of GTP to cyclic guanosine 3′,5′-monophosphate (cGMP). The role of cGMP is to activate downstream processes that ultimately lead to physiological effects. cGMP signaling can occur through three main mechanisms. The first and more prominent is through cGMP-activated protein kinases (PKGs or cGKs) (Denninger and Marletta, 1999), which mediate several processes such as endothelial permeability and vascular smooth muscle cell contraction. The second mechanism involves cGMP-regulated phosphodiesterase, which catalyze the generation of adenosine monophosphate (AMP) and GMP from cAMP and cGMP, respectively (Degerman et al., 1997). The third mechanism requires the activation of cyclic nucleotide-gated ion channels, which are non-specific cation channels involved in photo-transmision and olfaction (Mazzolini et al., 2018).

NO can also directly or indirectly induce post translational modifications of other proteins, which regulate their activity. These modifications include S-nitrosylation (Brown and Borutaite, 2004), S-glutathionylation (Adachi et al., 2004) and tyrosine nitration (Souza et al., 2008). Its primary modification, S-nitrosylation occurs when NO binds thiol in cysteines forming a S-nitrosothiol group, which induces changes in protein configuration and prevents their oxidation (Maron et al., 2013). As discussed above, S-nitrosylation is important for the negative feedback mechanism that controls eNOS function. Additionally, it can also inhibit caspases that trigger apoptosis (Li et al., 1997) or modify the function of hemoglobin and cardiac Ca^{2+} release channel (Xu et al., 1998).

Additionally, NO can compete with O_2 for oxidative phosphorylation during respiration at physiological concentrations (Clementi et al., 1999). Therefore, NO can inhibit mitochondrial respiration, and control apoptosis and generation of reactive oxygen species (ROS) (Erusalimsky and Moncada, 2007). Additionally, NO can also activate Ca^{2+}

dependent potassium channels in vascular smooth muscle cells (Bolotina et al., 1994) and trabecular meshwork cells (Dismuke and Ellis, 2009).

NO itself is not an oxidant, but it can easily be converted into more reactive molecules, known as reactive nitrogen species. For instance, NO can react with superoxide to produce peroxynitrite anion ($ONOO^-$), which in turn can act as a signaling molecule (Adams et al., 2015).

3. Nitric oxide in the vasculature

NO is involved in many aspects of normal physiology depending on the resident cell/tissues. For example, NO participates in the regulation of vascular tone, platelet aggregation, inflammation, neurotransmission and immune response. Concerning IOP regulation, we will focus on the roles that NO plays on both vascular physiology and pathophysiology, especially on the effects of endothelial-derived NO in vascular tone and permeability.

3.1. Normal vascular physiology

Vascular tone dictates the extent of tissue perfusion. NO is the most potent endogenous vasorelaxant, controlling vascular tone by relaxing vascular smooth muscle (VSM) cells that surround endothelial vessels. The tone of VSM is controlled primarily by Ca^{2+} levels, with high Ca^{2+} concentrations promoting contraction and low calcium promoting relaxation (Wu and Bohr, 1991). When Ca^{2+} levels in the cell are high, CaM activates myosin light chain (MLC) kinase (MLCK), which phosphorylates MLC promoting a cross bridge of the myosin head to actin filaments resulting in VSM contraction and vasoconstriction (Iida and Potter, 1986; Raina et al., 2009; Rembold and Murphy, 1993; Van Lierop et al., 2002; Walsh, 1994). However, in the case of low Ca^{2+} levels, MLC phosphatase dominates, mediating MLC dephosphorylation that breaks the cross bridge between the myosin head and the actin filament and results in cell relaxation/vasodilation (Nakamura et al., 2007). NO-regulated cell relaxation mostly occurs through cGMP/PKG signaling, which reduces Ca^{2+} levels through 3 main mechanisms: *i*) reduction of inositol triphosphate (IP_3)-mediated Ca^{2+} release from the sarcoplasmatic reticulum (Geiselhoringer et al., 2004; Xia et al., 2001), *ii*) increased Ca^{2+} sequestration by sarco/endoplasmic reticulum Ca^{2+}-ATPase activation (Raeymaekers et al., 1988) and *iii*) reduced Ca^{2+} entry by hyperpolarization due to K^+ channel opening and closure of Ca^{2+} channels (Chen et al., 2009; Murphy and Brayden, 1995; Robertson et al., 1993; Schubert et al., 2004; Tanaka et al., 2006). Additionally, NO can also trigger relaxation independently of cGMP through production of peroxynitrite that can activate SERCA (Adachi et al., 2004; Cohen et al., 1999) and by S-nitrosylation of proteins that control G-protein coupled receptors (Aronstam et al., 1995; Ignarro et al., 1981; Miyamoto et al., 1997). Moreover, NO inhibits the expression of endothelin-1 (ET1), a potent vasoconstrictor and physiological antagonist of NO that is important for controlling vascular tone (Haefliger et al., 1992). Importantly, NO and ET1 are potent regulators of trabecular meshwork (TM) tone, and thus outflow resistance (Dismuke et al., 2014).

Endothelial permeability is controlled through regulation of cellular junctions and cell contraction to allow for molecular movement through the vessel wall. Depending on the tissue's function, the basal level of permeability is different - while the blood brain barrier

forms a very tight endothelium, organs like the pancreas and SC have leakier endothelia (Claesson-Welsh, 2015; Overby et al., 2009; Ye et al., 1997). Studies with *NOS3* KO mice showed that eNOS appears not be involved in maintaining basal permeability (Fukumura et al., 2001; Hatakeyama et al., 2006). Nonetheless, it is required for mediating the response to external stimuli that would lead to the increase in permeability necessary for certain physiological processes (He et al., 1999; Marumo et al., 1999; Mayhan, 1999). Products such as VEGF and insulin-like growth factor, and shear stress trigger Akt signaling that promote eNOS activation through phosphorylation of Ser1177 (Feliers et al., 2005; Fleming et al., 2005), increased Ca^{2+} influx (Bates and Curry, 1997) and eNOS internalization (Sanchez et al., 2009). Downstream of NO, permeability is mediated through sGC and PKG (He et al., 1998; Yuan et al., 1993) that trigger either ERK-1/2 MAP (Kong et al., 2017; Varma et al., 2002) or cGMP/cAMP signaling (Cullere et al., 2005; Rangarajan et al., 2003). Ultimately, these cascades lead to reorganization of the cytoskeleton, focal adhesions, and intercellular junctions (Garcia et al., 1995; Goligorsky et al., 1999; Moy et al., 2004; Predescu et al., 2005; Stasek et al., 1992). NO is also directly involved in VE-cadherin phosphorylation and internalization (Di Lorenzo et al., 2013; González et al., 2003; Sandoval et al., 2001; Yang et al., 2015). As mentioned above, VE-cadherin is a foundational protein of the adherens, which regulates vascular permeability (Bazzoni and Dejana, 2004).

3.2. Endothelial dysfunction

Impaired NO signaling leads to endothelial dysfunction, which is implicated in the development of cardiovascular diseases, diabetes, metastasis in cancer, retinal diseases and glaucoma. Such diseases are usually multifactorial and emerge as a result of a defective homeostatic balance between vasodilation and vasoconstriction as well as compromised control of endothelial permeability.

Endothelial dysfunction is commonly associated with reduced NO bioavailability. The reasons for limited access to NO can be diverse and include both reduced eNOS expression and activity as well as increased NO scavenging (Duplain et al., 2001; Oemar et al., 1998; Tonduangu et al., 2004). Reduced eNOS activity can also be due to multiple factors such as, limited L-arginine availability (Schlaich et al., 2004), altered eNOS phosphorylation (Smith and Hagen, 2003; Wagner et al., 2007), eNOS uncoupling due to changes in CAV1 (Darblade et al., 2001), BH_4 (Topal et al., 2004) or hsp90 (Ou et al., 2003) expression, or eNOS inhibition by increased presence of asymmetric dimethylarginine (ADMA), a L-arginine analogue (Boger et al., 1998). In addition, the NO that is produced can be rapidly scavenged in the presence of ROS that promote the conversion to peroxynitrite, which can lead to further endothelial damage (Cassuto et al., 2014; Csiszar et al., 2002; Mohazzab et al., 1994; Scheuer et al., 2000; Sun et al., 2004). Lack of NO has been associated with multiple pathologies, for instance, insulin resistance in diabetes, muscular damage due to vasoconstriction, atherosclerosis, hypertension and acute myocardial infarction (Bian et al., 2008; Brenman et al., 1995; Su, 2015).

NO-mediated vascular hyperpermeability can be another cause of disease whereby NO abundance is elevated secondary to increased signaling of factors that stimulate eNOS

(Tilton et al., 1999). For example, overproduction of VEGF is responsible for the uncontrolled angiogenesis and leaky vessels seen in retinal diseases such as age-related macular degeneration and macular edema (Jirarattanasopa et al., 2012; Johnson, 2009; Penn et al., 2008) as well as in cancer, which contributes to metastasis (Fukumura et al., 2006).

The major risk factor for endothelial dysfunction is aging (Csiszar et al., 2002; Donato et al., 2011; Ungvari et al., 2010, 2018). Aged vasculature show signs of impaired eNOS/NO function, such as increased presence of ROS (Csiszar et al., 2002; Harman, 1956), loss of PI3K/Akt-dependent eNOS phosphorylation (Smith and Hagen, 2003), reduced autophagy (LaRocca et al., 2012), increased apoptosis (Csiszar et al., 2004) and reduction in production and sensitivity to systemic circulating factors that regulate eNOS activity (Mieno et al., 2006; Ryan et al., 2006). Similar processes are observed in response to other risk factors such as smoking (Barbieri et al., 2011; Celermajer et al., 1993; Golbidi et al., 2020; Jefferis et al., 2010; Su et al., 1998) and obesity (Gruber et al., 2008; Higashi et al., 2001). Aging (Gabelt and Kaufman, 2005; Liu et al., 2018; Rudnicka et al., 2006) is a primary risk factor for glaucoma.

Treatments for diseases caused by endothelial dysfunction aim to target the eNOS/NO system. For example, antioxidants such as Vitamin C and genistein confer protection against ROS (Heitzer et al., 1996; Matsumoto et al., 2003; Vera et al., 2007; Zhen et al., 2012) and eNOS expression can be enhanced by angiotensin-converting enzyme inhibitors (Bachetti et al., 2001; Fujii et al., 2002), phosphodiesterases-5-inhibitors (De Young et al., 2008; Salloum et al., 2003) and statins (Rikitake and Liao, 2005). Statins also stimulate eNOS activity by improving PI3K/Akt signaling (Kureishi et al., 2000) and promoting interaction with hsp90 (Feron et al., 2001) and BH_4 (Antoniades et al., 2011).

Clinical evidence also suggests that POAG patients exhibit features of impaired NO signaling. POAG patients demonstrate dysregulated retinal blood flow (Feke et al., 2014; Feke and Pasquale, 2008) and impaired brachial artery flow-mediated vasodilation (Fadini et al., 2010; Su et al., 2008), phenotypic features felt to be bioassays for NO signaling. Interestingly, several genes related to NO signaling have emerged as biomarkers for glaucoma or glaucoma related traits (GUCY1A3/B3, ITPR3, NOS3, EDNRB, CAV1; see Fig. 4). The question remains regarding how a polygenic risk score for impaired NO signaling based on these discovered glaucoma-related loci for glaucoma might be related to POAG on an individual basis.

4. NOS3/eNOS, nitrates and risk of glaucoma

4.1. Genetics

POAG is a common, complex disease with multiple target tissues including TM cells, SC endothelium, collector channels, ciliary body cells of several types, vascular endothelia that supply critical structures in the anterior and posterior segment, glial and other support cells and most importantly retinal ganglion cells (Fig. 4). Additionally, POAG is a highly heritable (Cuellar-Partida et al., 2016; Wang et al., 2017a) and polygenetic disease (Craig et al., 2020). Current genome-wide association studies report 127 genomic loci associated with POAG risk (Bailey et al., 2016; Choquet et al., 2018; Gharahkhani et al., 2020; Shiga et al.,

2018), 2 of which are unique to normal tension variant of POAG (Wiggs et al., 2012). Additionally, 112 loci are associated with elevated IOP (Gao et al., 2018; Khawaja et al., 2018; MacGregor et al., 2018), and many loci are associated with other POAG endophenotypes (vertical cup-disc ratio, and optic nerve cup area) (Choquet et al., 2020; Springelkamp et al., 2017; Wiggs and Pasquale, 2017). It is important to place loci related to NO signaling in context with all emerging variants related to POAG.

Genetic loci related to NO signaling play a central role in the functioning of all these tissues. While *NOS3* loci are not genome wide variants for POAG, their relation to the disease is modified by environmental influences including postmenopausal hormone use (Kang et al., 2010), oral contraceptive use (Kang et al., 2011a) and smoking history (Kang et al., 2011b). The functional significance of *NOS3* variants in relation to the glaucomatous process were supported when Stamer et al. (2011) reported that mice overexpressing *NOS3* had lower IOP and increased outflow facility than wild type, mice. Conversely, Lei et al. (2015) reported that the *NOS3* KO mice developed increased IOP and reduced outflow facility compared to wild type mice. As mentioned above, eNOS and CAV1 are biophysically juxtaposed in the caveolae of TM cells, which are critical for trans-cellular transport of aqueous humor from the anterior chamber into SC. The first meaningful and reproducible genome-wide variants associated with POAG were intergenic *CAV1/−CAV2* variants (Thorleifsson et al., 2010) and their relation to the glaucomatous process was asserted when Elliott et al. (2016) reported that CAV1 KO also developed elevated IOP. Protein products of *NOS3* and *CAV1* interact in complex ways to regulate aqueous humor outflow and their interplay in this process remains under investigation (Song et al., 2019).

Impaired NO signaling clearly produces unfavorable aqueous humor dynamics, and a critical question is whether impaired NO signaling can also produce progressive optic neuropathy. Buys et al. (2013) knocked out the alpha 1 subunit of sGC in a murine model and found a modest age-related increase in IOP with concomitant optic nerve degeneration. As mentioned, sGC is the most prominent intracellular receptor for NO (Buys et al., 2013). This same model, which shows notable similarities to human POAG, was also used to demonstrate that inhaled NO lowered IOP in a sGC-dependent manner (Muenster et al., 2017). Other common loci involved in endothelial cell function (Khawaja et al., 2018) that are also linked to POAG and are expressed in the outflow pathway include endothelial tyrosine kinase (*TEK,* or Tie2), *VEGFC,* and angiopoietin 2 (*ANGPT2,* a ligand for TEK) (Fig. 4). Interestingly, TEK is implicated in the morphogenesis of the outflow pathway (Thomson et al., 2017); in fact, rare loss-of-function variants in *TEK* are associated with congenital glaucoma (Souma et al., 2016).

4.2. Nutrition

The NO signaling pathway is a highly druggable pathway and is also one that could be favorably manipulated by dietary interventions (Fig. 5). Chuman et al. (2000) observed that an intravenous infusion of L-arginine led to a significant drop in IOP. In another study that investigated NO synthesis pathway metabolites in relation to POAG (Javadiyan et al., 2012), it was observed that while plasma L-arginine concentrations did not differ, there were significant increases in plasma ADMA, another NOS inhibitor, and symmetric

dimethylarginine concentrations in 211 advanced glaucoma cases compared with 297 controls ($P \leq 0.0001$). Consumption of nitrogen-fixating vegetables could be a ready source of NO via conversion of nitrates to nitrites and ultimately NO through a variety of enzymatic and nonenzymatic means (Fig. 5). Kang et al. (2016) postulated that this exogenous source of nitrates might reduce the risk of POAG. In fact, using data from two health professional cohorts, high dietary nitrate intake derived from vegetable sources was associated with a 21% reduced risk of incident POAG. Intake of lettuce (e.g., iceberg, romaine), spinach, and cruciferous vegetables (e.g., broccoli, kale, cabbage, cauliflower, brussels sprouts), celery, potatoes and onions accounted for 80% of nitrate intake from vegetables in this study. It is unknown whether a diet high in nitrates from vegetable sources might modify the course of glaucoma for patients with existing disease, particularly amongst patients with an overall high genetic burden of POAG risk variants.

5. Nitric oxide and aqueous humor dynamics

5.1. Nitric oxide effects on IOP and outflow facility in different species

The importance of eNOS activity and NO bioavailability on IOP homeostasis and outflow facility has been studied by either inhibiting or stimulating eNOS function or NO supplementation. Shown in Table 1 is a compilation of studies that have assessed the effect of NO on IOP and/or outflow facility. The strategies used to study the effect of NO on outflow facility and IOP range from genetic modifications that result in over-expression or KO of eNOS to pharmacological treatments that inhibit eNOS or donate NO. The often-used eNOS inhibitors are L-N^G-nitroarginine methyl ester (L-NAME) and cavtratin. Whereas NO activity is enhanced by NO donors (sodium nitroprusside or S-nitroso-N-acetylpenicillamine) or eNOS substrate (L-arginine). Additionally, the route of administration also changes between studies. Nonetheless, the consensus is that stimulation of eNOS activity results in improved outflow function and reduced IOP; while eNOS inhibition results in increased outflow resistance and IOP elevation (Table 1).

Different animal models are used to study aqueous humor dynamics and glaucoma (Bouhenni et al., 2012) and the different species are also represented when assessing the role of eNOS/NO on outflow regulation. The main difference between animal models is the anatomy of the outflow pathway. Primate and mouse eyes have a mature and continues SC with lamellated TM (Burgoyne, 2015; Dismuke et al., 2016; Overby et al., 2014a; Smith et al., 2001). In contrast, bovine, pigs, sheep and rabbit eyes exhibit a discontinuous and tortuous duct known as the angular aqueous plexus (Bergmanson, 1985; Lei et al., 2010). Our theoretical model would apply to those models with a true SC.

5.2. NOS expression/localization

In the iridocorneal angle tissues, all three NOS isozymes are expressed by the different cell types that participate in the regulation of IOP. Immuno-affinity studies of intact porcine tissues show that ciliary epithelial cells express nNOS and iNOS (Meyer et al., 1999) and expression is retained by non-pigmented epithelial cells in culture (Shahidullah et al., 2007). These findings were validated using a second method, whereby NADPH diaphorase staining (reflective of NOS activity) localized to ciliary epithelium of rabbit (Osborne et al., 1993).

Another study using a biochemical activity assay of dissected ciliary processes showed presence of a constitutive form of NOS (nNOS or eNOS) in bovine eyes (Geyer et al., 1997).

In the ciliary muscle (CM) of human eyes, NOS positive neurons were identified associated with anterior circular and reticular CM fibers, but not longitudinal muscle fibers using NADPH diaphorase staining (Tamm et al., 1995). In contrast, two other studies detected NOS activity in all three CM fiber types using the same method (Chen et al., 1998; Nathanson and McKee, 1995). These later findings of NOS activity in neurons associated with the longitudinal muscle fibers was called into question when a subsequent study found that only species with a fovea centralis express nitrergic neurons in the CM, thought to smoothen circular and reticular-mediated contractions during accommodation (Tamm and Lutjen-Drecoll, 1997).

In porcine ocular tissues, eNOS was confined to vascular endothelia including ciliary body vessels (SC and scleral vessels were not examined) (Meyer et al., 1999). Using eNOS-GFP reporter mice, eNOS expression was only observed in vascular endothelia of the ciliary body, SC and scleral vessels (Chang et al., 2015). There are conflicting reports of eNOS expression by ciliary epithelial cells (Meyer et al., 1999; Shahidullah et al., 2007). This contradiction may be related to antibody specificity and can soon be resolved with single cell RNA sequencing technology as seen for the TM.

Recent data has shed light on whether TM cells express NOS. NADPH diaphorase activity was detected in both the TM and SC in human eyes (Nathanson and McKee, 1995). By immunohistochemistry, nNOS was observed in all regions of TM (Meyer et al., 1999). Using an alternate method, a biochemical activity assay of dissected TM and ciliary body from bovine eyes suggest presence of a constitutive form of NOS, with TM/SC likely containing more than one subtype (Geyer et al., 1997). RNA expression of TM in human anterior segments showed an increased in iNOS, but not eNOS or nNOS after elevation of IOP (Schneemann et al., 2003). Recent breakthroughs in single cell RNA sequencing technology reveal in two independent studies of human outflow tissues that TM cells do not express any of the three NOS isoforms (Patel et al., 2020; van Zyl et al., 2020) (Table 2). van Zyl et al. (2020) also shows similarly negligible expression of NOS isoforms in TM of mice, which is noteworthy considering a large number of mechanistic studies are performed using mice. Positive labeling or activity may come from the abundant numbers of resident macrophages in the TM, which are known to produce NO (Camelo et al., 2004; Margeta et al., 2018; McMenamin and Holthouse, 1992; Patel et al., 2020).

5.3. Nitric oxide production by ciliary processes

Consistent with expression studies showing NOS enzymes in ciliary epithelia, isolated human and porcine ciliary processes produce NO (Haufschild et al., 2000). Morphine, working by activation of mu opioid receptors, raises cGMP levels and induces NO production in isolated iris-ciliary bodies (Dortch-Carnes and Randall, 2009). It appears that in addition to cGMP, cAMP is important in regulating NO production by ciliary epithelium. For example, drugs activating adenylyl cyclase such as forskolin, or cAMP analogues increase NO production by isolated porcine ciliary processes (Liu et al., 1998, 1999, 2002; Wu et al., 2003). In fact, in enucleated eyes perfused with epinephrine into their posterior

ciliary artery increased NO levels are detected at opening of TM by a NO probe (Millar, 2003). In contrast, ET1 decreases NO levels (basal or forskolin-stimulated) produced by isolated porcine ciliary processes (Wu et al., 2003), likely by inhibiting cAMP levels (Bausher, 1995).

5.4. NO/cGMP effects on aqueous humor production/ciliary epithelial transport

In anesthetized rabbits, inhibition of tonic NO production by intravenous L-NAME causes ciliary vessel constriction, and decreases aqueous humor flow; mathematically accounting for 66% of effects on IOP (Kiel et al., 2001). In contrast, perfusion of cyclic GMP, SNP or sodium azide into the ophthalmic artery of enucleated pig eyes reduces aqueous humor formation (Shahidullah et al., 2005). Thus, there appears to be NO effects both on blood flow to the ciliary body, and transport of ions and water across the ciliary epithelia.

Depending upon the experimental paradigm, NO-GC-cGMP activity decreases or increases ion and water transport. For example, in isolated porcine ciliary processes activation of the NO-GC-cGMP pathway depolarizes ciliary epithelial transmembrane potential, likely by stimulating stroma-to-aqueous anionic transport (Fleischhauer et al., 2000; Wu et al., 2004). Similarly, cGMP reverses cAMP-mediated inhibition of the Na^+/K^+-ATPase pumps at the aqueous surface of both rabbit and cat ciliary epithelia using whole-cell patch clamping and increasing K^+-channel activity, thus driving pump activity of the rabbit pigmented epithelial cells at the stromal surface (Carre and Civan, 1995). In contrast, NO-mediated increase in cGMP and PKG-dependent Src family kinase activation (Shahidullah et al., 2014) inhibits Na^+/K^+-ATPase activity in cultured non-pigmented epithelial cells (Shahidullah and Delamere, 2006).

5.5. Nitric oxide in aqueous humor of normal vs. glaucoma

Several studies have measured the amount of NO in aqueous humor of patients suffering from different types of glaucoma, using cataract patients as their control group. As displayed in Table 3, there is no general agreement on the standard values of NO in aqueous humor in health and disease. Regardless, control eyes appear to contain NO within the range of 20–100 μmolar. It is important to mention that using subjects with cataracts as controls may be a confounding aspect itself, and may not be representative of healthy population (Kao et al., 2002).

Concerning the NO values in POAG, the reports do not agree: Of the 6 studies we found, 2 report no change, 2 report a significant increase and 2 report a significant decrease in comparison to cataract. These studies should be interpreted cautiously as the authors report that the development state of the disease, IOP of the subjects at the time of analysis and treatment regimens are limitations in their studies that may affect the results. Furthermore, these discrepancies may come from differences in study designs of the studies, sample sizes and, principally, the analytical tools used to measure NO, as some studies measure nitrite as a surrogate for NO. Moreover, these issues are complicated by the reality that NO is short lived and reactive, thus probably not a good biomarker of disease.

Regarding the other types of glaucoma, the elevated NO levels observed in neovascular glaucoma may be secondary to the increased presence of VEGF in aqueous humor of

patients with this pathology (Tripathi et al., 1998; Wang et al., 2015a; Aiello et al., 1994). As per angle closure glaucoma, Chang et al. (2000) acknowledge that their patients had underwent surgery to lower IOP and showed signs of inflammation, that could result in NO production. Similar concerns about chronic inflammation are risen by Ghanem et al. (2011). Interestingly, they also suggest that producing NO might be a natural mechanism for the eye to lower IOP, but that it fails due to a closed irideocorneal angle.

5.6. NO and unconventional and conventional outflow function

In organ bath, exogenous NO relaxes precontracted longitudinal bovine (Beauregard et al., 2001; Wiederholt et al., 1994) and monkey (Gabelt et al., 2011) CM strips. However, when CM fibers are relaxed by atropine *in vivo*, outflow facility decreases (Kiland et al., 1997). This is consistent with (*i*) anatomical connection of the CM and TM via an elastin fiber network and (*ii*) the contractile dominance of CM over TM in intact systems (Wiederholt et al., 2000). Since NO lowers IOP, NO appears to preferentially relax the TM compared to CM (Heyne et al., 2013; Schuman et al., 1994). In fact, in *ex vivo* studies that functionally isolate the conventional outflow pathway, NO donors increase, and NOS inhibitors decrease outflow facility (Dismuke et al., 2008; Schneemann et al., 2002). Moreover, direct activation of sGC or perfusing exogenous NO in enucleated mouse eyes also increases outflow facility (Chang et al., 2015; Ge et al., 2016). Lastly, outflow facility is double that of wild type littermates in transgenic mouse eyes overexpressing eNOS in SC endothelia (Stamer et al., 2011), whereas outflow facility was diminished by 30% in eNOS KO mice (Lei et al., 2015).

NO appears to have two primary sites of action in the conventional tract: cells of the juxtacanalicular tissue (JCT) and smooth muscle-containing vessels distal to SC (e.g. collector channels and intrascleral venous plexus). In the JCT, NO relaxes TM cells (Dismuke et al., 2014; Wiederholt et al., 1994) and reduces their cell volume (Dismuke et al., 2008; Ellis et al., 2010), which alters conventional outflow tissue geometry and flow passages for aqueous humor to increase outflow. Also in the JCT, NO is hypothesized to increase the permeability of SC endothelium by promoting the disassembly of cell-cell junctions (see below) and has been shown to decrease cell volume (Dismuke et al., 2008). Together, these two mechanisms are thought to open paracellular flow passageways, or so-called “B-pores” (Ethier et al., 1998). To functionally isolate the second site of NO action (the vasoactive vessels distal to SC), the TM in human and porcine anterior segments was removed by trabeculotomy and distal vessels kept viable in perfusion organ culture. Under this paradigm, NO significantly increases outflow facility and alters the diameter of distal vessels (McDonnell et al., 2020; Waxman et al., 2018). This behavior is consistent with location of nitrergic neurons that terminate on these intrascleral veins distal to SC, likely innervating associated smooth muscle cells (Overby et al., 2014a).

5.7. TM contractility

The TM has smooth muscle-like contractile properties, as many TM cells express contractile proteins such as α-smooth muscle actin and myosin, although the TM is a heterogenous tissue and not all cells have the same properties (Coroneo et al., 1991; de Kater et al., 1990, 1992; Ko and Tan, 2013; Stamer and Clark, 2017). Accordingly, TM contractility can be modulated by NO (Dismuke et al., 2014). Furthermore, TM contraction has been associated

with reduced aqueous humor outflow (Bertrand et al., 2020; Wiederholt et al., 1995) while TM relaxation increases outflow (Rao et al., 2001; Zhang and Rao, 2005), both affecting IOP (Luna et al., 2012). Similar to VSM cells, targeting PKC to activate MLCK relaxes TM cells affecting outflow facility and IOP (Rao et al., 2005; Tian et al., 2009). NO also relaxes the TM through cGMP signaling (Wiederholt et al., 1994) and Ca^{2+}–dependent maxi-K^+ channels (Stumpff et al., 1997). Additionally, genes regulating vascular tone are associated with POAG (Kang et al., 2014).

On the other hand, molecules that are elevated in aqueous humor of glaucomatous eyes such as TGF-β and ET1 alter TM tone (Choritz et al., 2012; Cousins et al., 1991; Junglas et al., 2012). ET1 contracts the TM of bovine eyes in a Ca^{2+}–independent manner through the GTPase Rho and ROCK signaling (Rao and Epstein, 2007; Renieri et al., 2008; Thieme et al., 2000), as a consequence, ROCK inhibition relaxes the TM (Nakajima et al., 2005; Tanihara et al., 2008). Additionally, loss of α-smooth muscle actin in the TM is associated with aging (Flügel et al., 1992).

5.8. SC permeability

SC is a leaky endothelium but is also responsible for forming part of the blood-aqueous barrier that keeps blood products from entering the anterior chamber. Aqueous humor crosses SC's continuous endothelium containing tight junction through either paracellular (border pores or B-pores) or intracellular pores (I-pores) to access the canal's lumen (Braakman et al., 2016; Epstein and Rohen, 1991; Ethier et al., 1998), with B-pores providing the main fluid path (Braakman et al., 2015). The integrity and hydraulic conductance of SC is maintained and regulated through the expression of tight junction proteins (mostly Claudin-11, ZO-1 and tricellulin) (Alvarado et al., 2004; Bhatt et al., 1995; Raviola and Raviola, 1981; Tam et al., 2017) and adherens junction proteins (mostly VE-cadherin and PECAM-1) (Heimark et al., 2002; Perkumas and Stamer, 2012). SC permeability is clearly a contributing factor to outflow resistance generation and may be compromised in disease. For instance, pore density is reduced in glaucoma (Allingham et al., 1992; Johnson et al., 2002) and inhibition of tight junctions in SC results in increased outflow facility (Ethier and Chan, 2001; Tam et al., 2017). Moreover, cell junctions change with changing pressure (Burke et al., 2004; Ye et al., 1997), suggesting dynamic regulation in response to IOP. Products known to increase endothelial permeability such as NO (see Table 1), VEGF (Reina-Torres et al., 2017), and Rho-GTPase inhibitors (Lu et al., 2008; Rao et al., 2001) increase outflow facility. On the contrary, products that reduce endothelial permeability like dexamethasone (Overby et al., 2014b; Underwood et al., 1999), Rho-GTPase activators (Kumar and Epstein, 2011) and pigment epithelium-derived factor (Rogers et al., 2013) decrease outflow. NO is likely involved in the effects observed in response to these products but there is no direct evidence in outflow tissues (Brook et al., 2019; Igarashi et al., 2013; Mayhan, 1999; Ming et al., 2002; Sugimoto et al., 2007). S1P, despite being an eNOS activator (Igarashi and Michel, 2008), is known to reduce endothelial permeability (McVerry and Garcia, 2005). As such, treatment with S1P increases peripheral actin in human SC cells, suggesting increased junctional assembly (Sumida and Stamer, 2010) and reduces outflow facility (Boussommier-Calleja et al., 2012; Mettu et al., 2004; Stamer et al., 2009).

Studying the specific role of eNOS/NO on SC permeability and their effect on outflow resistance regulation is not easy. As already mentioned, NO produced by SC cell can have autocrine and paracrine effects; therefore, the physical proximity between SC and JCT makes it difficult to separate their relative effects. An alternative testing strategy would be to perform *in vitro* tests on isolated SC cells in culture. However, cultured SC cells do not form proper cell junctions (Johnson, 2006; Perkumas and Stamer, 2012; Stamer et al., 1998). Hence, traditional permeability assays and measurements of hydraulic conductance are not as informative for SC physiology as they are for endothelial monolayers that form better cell junctions in culture.

5.9. NO transport within the conventional outflow pathway

As a gasotransmitter, NO is a labile molecule with a very short half-life on the order of seconds in vascularized tissues. This relatively short half-life is due to its interaction with heme, and hemoglobin in particular, which is the main physiological quencher for NO activity (Beckman and Koppenol, 1996). However, the conventional outflow pathway is avascular and, with the exception of when a hyphemia is present, virtually free of hemoglobin. In heme-free aqueous environments, the half-life of NO is typically determined by its reaction rate with dissolved oxygen species, such as O_2 or superoxide (Beckman and Koppenol, 1996). When reacting with O_2, NO is predicted to have a half-life on the order of minutes (Ford et al., 1993; Hakim et al., 1996; Lewis and Deen, 1994) or longer because the half-life of NO increases at lower concentrations of O_2 (Beckman and Koppenol, 1996). The reaction between NO and superoxide occurs much faster, but superoxide itself is a toxic free radical that is buffered by antioxidant enzymes such as superoxide dismutase (Beckman and Koppenol, 1996), which are abundant in the ocular anterior segment. In the relatively hypoxic anterior chamber where the partial pressure of O_2 is typically <25 mmHg (as opposed to 150 mm Hg for room air) (Siegfried et al., 2010), superoxide levels are likely to be low. Thus, while it remains uncertain exactly how long NO persists within the conventional outflow pathway, the absence of hemoglobin and the relatively hypoxic aqueous environment suggests that NO will almost certainly persist for longer than it does in vascularized tissues, possibly up to several minutes.

The relatively long half-life of NO in aqueous humor has a number of important consequences for NO transport within the conventional outflow pathway. Firstly, any NO that is produced by SC cells is available for diffusion/transport to the TM, collector channels or elsewhere in SC, where it may have a bioactive effect. To appreciate this, we may calculate that the residence time for aqueous humor passing through the lumen of SC, which can be shown to be on the order of seconds (Braakman et al., 2016). This means that NO would have little time to react with dissolved oxygen, which occurs over the time scale of minutes, before reaching the collector channels. NO may also diffuse *upstream* relative to the direction of aqueous humor outflow, reaching the contractile TM or JCT cells. Diffusion of NO in the upstream direction is justified based on the value of the Péclet number, which for appropriate parameter values[2] is of order 0.1, indicating that the rate of diffusive

[2]The Péclet number (*Pe*) is defined as $Pe = u\,l/D$, where $u = 3\ \mu m/s$ is the velocity of aqueous humor flow through the TM, $l = 100\ \mu m$ is the TM thickness, and $D = 3 \times 10^{-5}\ cm^2/s$ is the diffusivity of NO, Zacharia and Deen, 2005.

transport of NO across the TM is roughly 10-fold faster than the rate of advective transport. Diffusion of NO across the TM occurs within seconds, faster than the NO decay rate. This allows NO produced by SC cells to reach TM cells despite the continual flow of aqueous humor through the outflow pathway.

Alternatively, any hemoglobin that becomes present within the outflow pathway will quickly deplete any available NO. The most common cause for such a scenario is blood reflux into SC lumen that may occur during periods of hypotony or elevated episcleral venous pressure (EVP). As NO regulates outflow resistance generated both within the TM and in distal vessels, it is tempting to speculate that blood reflux, by introducing hemoglobin and depleting NO, may act as a physical stimulus to increase outflow resistance and thereby oppose the reduction in IOP relative to EVP. Such a mechanism could potentially balance overproduction of NO.

6. Mechanisms of IOP mechanosensation and homeostasis in the conventional outflow pathway

Any model of IOP homeostasis requires a mechanism to sense and respond to changes in IOP. Mechanosensory mechanisms for IOP have been mainly attributed to IOP-induced stretch in the TM or shear stress due to circumferential aqueous humor flow in SC, although alternative models such as mechanosensitive nerve endings in the scleral spur (Tamm et al., 1994) have been proposed. As the bulk of outflow resistance generation lies within the outer TM, mechanisms for IOP mechanosensation within the TM and SC have the important consequence that they place the sensors and effectors of IOP change within the same anatomical location, allowing for the possibility of local regulation of outflow resistance.

6.1. Mechanosensing of outflow cells

Cells of the conventional outflow pathway reside within a demanding mechanical environment. Mechanical forces that act on outflow pathway cells arise due to pressure gradients and forces exerted by fluid flow. These mechanical forces are likely to vary regionally due to the non-uniform distribution of aqueous humor drainage, known as segmental outflow, because regions of higher local outflow may experience greater forces than regions of lower outflow. Furthermore, because of the ocular pulse transmitted by the cardiac cycle, these mechanical forces are highly oscillatory (~1 Hz in humans and 10 Hz in mice). Individual cells may also experience forces that depend on cell alignment or forces that vary directionally depending on the properties of the local tissue microenvironment. Thus, outflow pathway cells experience dynamic, spatially varying and multi-directional mechanical forces that arise due to the pressure drop and fluid drainage through the outflow pathway (Stamer et al., 2015).

Despite this complexity, the mechanical forces acting on outflow pathway cells can be broadly classified into two types: stretch and shear stress. Stretch arises due to tensional forces within the tissue, while shear stress arises due to the frictional forces associated with fluid flow. Outflow cells use multiple types of mechanosensors to detect stretch and shear stress. This includes Ca^{2+}-dependent and Ca^{2+}-independent mechanisms, caveolae and

CAV1, PIEZO1, the VEGFR/VE-cadherin/PECAM-1 complex, the Tie2/PI3K/Akt signaling pathway, and the glycocalyx. In this section, we first review how different mechanosensors may influence the response to TM and SC cells to stretch and shear stress and their potential relationship to outflow function.

6.2. Caveolin-1

As mentioned above, caveolae play a key role in the mechanosensation within the cardiovascular system and may have a similar mechanosensory role in the eye (Gu et al., 2017). One consequence of endothelial cells exposed to stretch, such as occurs with giant vacuole formation, is flattening and subsequent disassembly of caveolae (Dulhunty and Franzini-Armstrong, 1975; Lee and Schmid-Schonbein, 1995; Parton, 2018; Sinha et al., 2011). There is an abundance of caveolae expressed in cells of the outflow pathway such as the SC and the TM (Herrnberger et al., 2012; Tamm, 2009), and polymorphisms at the CAV1/2 gene loci have been reproducibly implicated in POAG and ocular hypertension (Chen et al., 2014; Huang et al., 2014; Hysi et al., 2014; Kim et al., 2015; Loomis et al., 2014; Ozel et al., 2014; Thor-leifsson et al., 2010; van Koolwijk et al., 2012; Wiggs et al., 2011). Furthermore, using CAV1 KO mice, a functional link between CAV1 expression, IOP, and conventional outflow dysfunction has been elucidated (Elliott et al., 2016; Kizhatil et al., 2016; Lei et al., 2016). In CAV1 KO mice, IOP and conventional outflow resistance are elevated despite concomitant increase in eNOS activity (Elliott et al., 2016). In endothelium specific CAV1 KO mice, IOP is still elevated but CAV1 expression in the TM is sufficient to rescue conventional outflow defects reported in global CAV1 KO mice (De Ieso et al., 2020). Additionally, external elevation of IOP increases eNOS activity in WT but not endothelium-specific CAV1 KO mice, where eNOS activity is abnormally elevated, suggesting CAV1 and caveolae play a mechanosensory role in the SC and distal vessels necessary for pressure-induced eNOS activation (De Ieso et al., 2020). Hyperactivity of eNOS was also evident as both global and endothelial CAV1 KO mice were more sensitive to reduced outflow facility by treatment with eNOS inhibitor, L-NAME (De Ieso et al., 2020; Elliott et al., 2016). Thus, it was postulated that eNOS hyperactivity secondary to CAV1 KO might partially compensate for another functional deficit in the conventional outflow pathway caused by caveolae deficiency (Fig. 6). One possible cause for functional deficit in the conventional outflow pathway could be enhanced production of NO-derived oxidants due to hyperactive eNOS (Lei et al., 2016; Song et al., 2017, 2019). A modest increase in protein nitration was observed in global and endothelium-specific CAV1 KO mice (De Ieso et al., 2020), however more investigation is needed to determine the level of nitrative stress in the conventional outflow tissues. Nevertheless, it is likely that loss of endothelial CAV1 and caveolae expression in the SC results in reduced shear stress-induced mechanosensation and regulation of eNOS signaling, similar to the mechanism described in Yu et al. (2006).

6.3. PIEZO1

PIEZO1 is expressed in SC of human eyes (van Zyl et al., 2020), and the TM of human (Tran et al., 2014) and mouse eyes (Morozumi et al., 2020), and there is some evidence to suggest that elevated IOP modulates PIEZO1 expression in mice (Ho et al., 2014). Additionally, it was recently shown that PIEZO1 transduces tensile stretch, shear flow, and

pressure in the TM. (PMID: 33226641). It would be interesting to further investigate the mechanosensory role of PIEZO1 channels in the regulation of IOP and outflow resistance, specifically, whether PIEZO1 regulates eNOS activity in SC cells, as observed in endothelial cells.

6.4. VEGFR/VE-cadherin/PECAM-1 complex

There are several reasons why this VEGFR/VE-cadherin/PECAM-1 complex located in cell-cell junctions could also have physiological implications in the regulation of shear-induced eNOS activity in the conventional outflow pathway. First, the majority of resistance to aqueous humor outflow occurs at the juxtacanalicular region or at the inner wall of the SC (Grant, 1963), and this resistance is partly mediated by the intercellular junctions at or near the inner wall of SC (Heimark et al., 2002). Second, the SC has a discontinuous basal lamina and SC cells might therefore likely rely on the integration of signals between integrins and cell-cell junctions (Heimark et al., 2002; Johnstone, 1979). Third, cell-cell junctions enable mechanical transduction between cells and allow the outflow tissues to function as a unit (Heimark et al., 2002; Wiederholt et al., 2000). Fourth, junctions in SC are very labile and pressure-dependent (Ye et al., 1997), and are a likely pathway of most fluid flow (Braakman et al., 2015; Ethier and Chan, 2001). Finally, human SC cells express PECAM-1, VE-cadherin (Heimark et al., 2002) and VEGFR-2 (Kizhatil et al., 2014), and direct activation of VEGFR-2 increases outflow in mice (Reina-Torres et al., 2017). SC cells also express VEGFR-3 (Aspelund et al., 2014), which is significant because SC is a dual blood and lymphatic vessel (Kizhatil et al., 2014), and VEGFR-3 is usually only expressed in lymphatics (Kaipainen et al., 1995). Thus, it is likely that the VEGFR/VE-cadherin/PECAM-1 complex regulates shear-induced eNOS signaling in the SC like in vascular endothelial cells, however further investigation is needed to test this hypothesis.

6.5. Tie2/PI3K/Akt

Like blood vascular endothelial cells, SC but not TM cells also express Tie2 (Kizhatil et al., 2014). Impaired ligand-dependent Tie2 signaling disrupts SC integrity and triggers POAG-associated pathogenesis, and Tie2 reactivation rescues POAG phenotype and rejuvenates SC in aged mice (Kim et al., 2017). It is not known whether Tie2 signaling plays a mechanosensory role in the SC; further investigation is required to determine whether shear stress induces the Tie2/PI(3)K/Akt pathway in the SC, and whether this mediates downstream eNOS activity. Regardless, Tie-2 activation in SC results in increased outflow facility and decreased IOP (Li et al., in press).

6.6. Glycocalyx

As in vascular endothelial cells, the glycocalyx is also present in the human conventional outflow pathway surrounding TM, SC, within most pores of SC endothelium, and coating the inner membrane of giant vacuoles with visible pore collector channels (Yang et al., 2014). It is theorized that the glycocalyx in SC plays a role in sensing and responding to shear stress, particularly with regard to shear-induced eNOS activity. Future work might involve investigating the effect of enzymatic degradation of glycocalyx-specific glycosaminoglycans on shear-induced NO production in the SC, in order to confirm whether

the glycocalyx plays a role in sensing and responding to shear stress in the SC as observed in vascular endothelium.

6.7. Stretch-mediated mechanosensation of IOP

As IOP increases, a mechanical force is exerted on the TM and inner wall of the SC as the cells of the TM and JCT stretch and expand toward SC's outer wall (Grierson and Lee, 1974; Johnstone and Grant, 1973; Moses, 1977; Sherwood et al., 2019; Van Buskirk, 1982). Mechanical stretch of the TM induces the production of several factors by TM cells that modulate outflow resistance (reviewed by WuDunn (2009)) such as matrix metalloproteases (MMPs) (Bradley et al., 2001), adenosine (Wu et al., 2012), and VEGF (Reina-Torres et al., 2017). Like the TM, the SC inner wall is constantly exposed to variable and transient stretching (Ethier, 2002), and increases of up to 50% have been recorded in monkey eyes where IOP was increased from 8 to 30 mmHg (Grierson and Lee, 1977; Overby, 2011). Mechanical stretch applied to SC cells may trigger pore formation to potentially contribute to outflow resistance generation (Braakman et al., 2014; Johnson et al., 1992). Finally, there is one study showing that elevation of pressure in perfused human anterior segments increased nitric oxide production that was coincident with increased iNOS expression, possible by resident macrophages (PMID: 12719994)).

The first model of IOP homeostasis was based on IOP-induced stretch in the TM and was proposed by Bradley et al. (2001). In this study, Bradley et al. (2001) exposed human anterior segments to elevated mechanical forces imposed by doubling the perfusion flow rate. In response to the doubling the flow rate, IOP initially doubled as expected for a constant resistance system. However, over several days, outflow resistance decreased, and IOP returned to near baseline values (Bradley et al., 2001), with a continual time-dependent increase in outflow facility (Acott et al., 2014). The change in outflow resistance coincided with an increased MMP production, suggesting that extracellular matrix remodeling within the TM is responsible for the change in outflow resistance (Bradley et al., 2001). The response appeared to be mediated by stretch because a similar increase in MMP expression was observed in stretched human TM cells. Importantly, this homeostatic mechanism appears to be disrupted in glaucomatous anterior segments (Raghunathan et al., 2018), suggesting that ocular hypertension in glaucoma may be associated with impaired IOP homeostasis.

6.8. Shear stress-mediated mechanosensation of IOP

The shear-mediated model of IOP mechanosensation was proposed by Stamer et al. (2011) (Fig. 7). According to this model, IOP-induced collapse of SC leads to increased shear stress acting on SC cells. The increase in shear stress results from the circumferential flow of aqueous humor that must pass through SC lumen in route to a collector channel ostium. As the lumen of SC narrows, the hydraulic resistance to flow through SC increases, leading to an increase in the frictional force acting on SC cells. Using fluid mechanical principles and building on an earlier mathematical model, Ethier et al. (Ethier et al., 2004; Johnson and Kamm, 1983) predicted the range of shear stresses acting on SC endothelial cells (Fig. 8), and a similar relationship was shown for mice (Stamer et al., 2011). The model considered SC to be elliptical in cross-section, with semi-major and semi-minor axes a and b,

respectively. With increasing IOP, the pressure drop across the TM and inner wall increases in response to an increasing force that constricts SC lumen and decreases *b*. As *b* decreases, the shear stress acting on SC cells increases non-linearly (Fig. 8). For smaller values of *b*, the SC shear stress increases sharply and reaches values of wall shear stress experienced in large arteries (Fig. 8). This work demonstrates how changes in IOP may be sensed via changes in shear stress acting on SC endothelium. As SC endothelial cells are shear-sensitive, this provides a mechanism by which changes in IOP may affect SC cell mechanobiology to modulate outflow function, allowing a potential homeostatic mechanism.

Human SC cells express eNOS (Perkumas and Stamer, 2012), and elevated shear stress in the SC triggers NO production by endothelial cells (Ashpole et al., 2014) similar to other human vascular endothelia (Davies, 1995). Shear stress also stimulates endothelial cell alignment near collector channels (Ashpole et al., 2014; Ethier et al., 2004). As detailed in the sections above, NO lowers outflow resistance and IOP (Table 1) via several mechanisms, including relaxation of the TM (Dismuke et al., 2014; Wiederholt et al., 1994), dilation of distal vessels (McDonnell et al., 2018) or increasing the permeability of SC inner wall as for vascular endothelia (Durán et al., 2013). Evidence for shear-mediated NO production in human anterior segments was recently provided by McDonnell et al. (2020) who reported that increased IOP leads to elevated nitrite production in the collected perfusate, consistent with increased NO production. Thus, shear induced NO production by SC cells is stimulated in response to IOP elevation. As NO is known to lower outflow resistance and thereby oppose the increase in IOP, all the pieces of a putative pathway are present to allow NO to function as a mechanosensitive regulator of IOP homeostasis. As discussed above, NO produced by SC cells is able to reach other outflow pathway tissues, including the TM and collector channels, where it may exert a bioactive response that modulates outflow resistance such as dilation of distal vessels. More investigation is needed to determine whether dilation of distal vessels can occur as a result of NO release from shear stress responses to fluid flow in the distal vessels, or solely from local neural responses. Parameters pertaining to shear stress-induced regulation of aqueous humor outflow that do not involve NO signaling are explored in further detail in the report by (Carreon et al., 2017).

6.9. Pulsatile mechanical forces in the outflow pathway: the effect of the ocular pulse

IOP is not static, but rather oscillates with a sinusoidal waveform having an amplitude of several mmHg. These pressure oscillations arise from pulsatile changes in choroidal blood volume over the cardiac cycle, known as the ocular pulse (Coleman and Trokel, 1969). This pulsatile pressure environment induces pulsatile shear stress in SC and pulsatile strain in the TM. To determine the magnitude of these pulsatile forces, Sherwood et al. (2019) developed a mathematical model of fluid flow through the TM and SC, building upon the previous model of Johnson and Kamm (1983) and accounting for the non-linear relationship between IOP and SC height (modelled as a rectangular channel). To mimic the effect of ocular hypertension, Sherwood et al. (2019) varied the outflow resistance attributable to the inner wall/JCT, r_{je}, which has a value of 2.08 mmHg/(μl/min) in a normotensive eye (Fig. 9). Increasing r_{je} led to an approximately linear increase in IOP and in the ocular pulse amplitude (indicated by the shaded regions in Fig. 9A). The predicted shear stress in SC also increased with r_{je}, curving upwards and becoming highly pulsatile, particularly for values of

r_{je} greater than approximately 5 mmHg/(μl/min) corresponding to ocular hypertensive conditions (blue shaded curve in Fig. 9B). Likewise, TM stretch also increased with r_{je}, but the relationship curved downwards and exhibited a much lower pulsatile magnitude relative to SC shear stress (blue shading in Fig. 9C).

6.10. Differential sensitivities of TM strain and SC shear stress to perturbations in outflow resistance

The mathematical predictions of Sherwood et al. (2019) support the notion that the outflow pathway exploits multiple mechanosensory mechanisms to detect changes in IOP. This can be appreciated based on the shape of the relationships describing how SC shear stress and TM stretch change as a function of r_{je} (Fig. 9). More specifically, the slope of these relationships represents the sensitivity of TM stretch or SC shear stress to perturbations in outflow resistance. Note that these slopes vary depending on r_{je}, indicating that the sensitivity of TM stretch or SC shear stress also varies depending on whether the eye is normotensive ($r_{je} \approx 2$ mmHg/μl/min) or hypertensive ($r_{je} > 5$ mmHg/μl/min, corresponding to IOP > 21 mmHg).

TM strain appears to be more sensitive to perturbations of outflow resistance under normotensive conditions. This is because the slope of the TM strain versus r_{je} relationship is significant around $r_{je} \approx 2$ mmHg/(μl/min), but the slope continually decreases for further increases in r_{je}. The amplitude of the pulsations in TM stretch is small under normotensive conditions and increases slightly with r_{je}, but even for the highest values of r_{je}, this amplitude never exceeds 7% of its time-averaged value. This is consistent with the relatively small amplitude of oscillatory displacement (typically < 1 μm) measured at the outer margin of the TM in living human eyes by phase-contrast OCT (Li et al., 2013; Xin et al., 2017, 2018).

The model suggests a progressive de-sensitization of TM stretch to further perturbations in r_{je} once outflow resistance becomes elevated. In other words, under ocular hypertensive conditions, TM stretch becomes *less* responsive to further elevations in outflow resistance or IOP. An alternative way to make this same point is to recognize that TM stretch reaches an upper limit once the SC lumen approaches complete collapse. This suggests that TM strain would function poorly as an IOP sensor under conditions when IOP was already elevated.

In contrast to the behavior observed for TM stretch, SC shear stress becomes *more* sensitive to perturbations in outflow resistance under ocular hypertensive conditions. As r_{je} increases, the slope of the SC shear stress relationship increases continually, indicating heightened sensitivity to further perturbations in outflow resistance, with the slope increasing sharply for $r_{je} > 5$ mmHg/μl/min. With increasing r_{je}, SC shear stress also becomes highly pulsatile, with the amplitude of the shear stress oscillations being comparable to its mean value (dark central curve in Fig. 9B). This suggests that the shear stress in SC may provide a sensitive means to detect further perturbations in outflow resistance and IOP under hypertensive conditions. The pulsations may also amplify the response of SC cells to shear stress, relative to that of steady conditions, as discussed below.

The findings by Sherwood et al. (2019) suggest that the outflow pathway may exploit different mechanosensory mechanisms that are tuned to different ranges of outflow resistance. Importantly, this would allow the outflow pathway to compensate for decreasing sensitivity on the part of one mechanosensory mechanism stimulus with increasing sensitivity of the other, to provide more robust IOP mechanosensation across a broader range of normotensive and hypertensive conditions. Further, the differential sensitivities of TM stretch and SC shear stress to r_{je} would allow the outflow pathway to differentiate small versus large perturbations in outflow resistance, and likely activate different mechanotransduction pathways. By exploiting multiple mechanosensory and mechanobiological mechanisms, the outflow pathway may thereby achieve a more robust homeostatic regulation of IOP that allows fine-tuning of outflow resistance over a wide range of physiological or supraphysiological conditions.

6.11. Increased TM stiffness inhibits IOP mechanosensation in the outflow pathway

Increased tissue stiffness is linked with the pathogenesis of several diseases including atherosclerosis (Palombo and Kozakova, 2016), fibrotic disease (Wells, 2013; Wynn and Ramalingam, 2012), and glaucoma (Last et al., 2011; Overby et al., 2014c; Vahabikashi et al., 2019; Wang et al., 2017b). TM stiffness is defined as the propensity for the TM tissue to resist deformation when a force or load is applied to it, such as elevated IOP or stretch (Wang et al., 2017b). There are currently two methods for direct measurement of tissue stiffness; compression (indentation) testing and tensile testing. As stated by Wang et al. (2017b), the actual value of the tissue stiffness measurements obtained from either of these two methods should be interpreted as a general indication of tissue stiffness, which might not be relevant in all situations, such as *in vivo*. However, if experimental conditions are kept constant, direct measurement of tissue stiffness, via compression or tensile testing, is still useful if used for relative comparisons of tissue stiffness between comparable samples (such as glaucomatous vs. age-matched, healthy). As determined by compression testing, a two-fold increase in stiffness was recorded in the TM of glaucomatous eyes compared to normal human eyes (Vahabikashi et al., 2019), roughly comparable with observations from Wang et al. (2017a). A much larger increase in TM stiffness of glaucomatous human eyes (20-fold) was observed by Last et al. (2011), however this large increase had been suggested to be attributable to differences in apparatus used for measuring tissue stiffness (Vahabikashi et al., 2019). TM stiffness is an important target in glaucoma therapy because drugs and factors that decrease TM stiffness such as Rho kinase inhibitors (Li et al., 2016; Ren et al., 2016) and NO (Dismuke et al., 2008) concomitantly reduce outflow resistance. TM cells relax when exposed to NO (Dismuke et al., 2014; Wiederholt et al., 1994). Additionally, factors that increase TM stiffness such as dexamethasone also increase outflow resistance and elevate IOP (Li et al., 2019; Raghunathan et al., 2015).

Despite studies showing that increased TM stiffness coincides with increased outflow resistance (Wang et al., 2017b, 2018), it remains unclear how TM stiffness may affect outflow function. The model of Sherwood et al. (2019) predicts that TM stiffness is key a factor controlling IOP mechanosensation, and elevated TM stiffness may inhibit IOP mechanosensation. In their mathematical model, Sherwood et al. (2019) considered the effects of increasing TM stiffness on TM stretch and SC shear stress. Specifically, they

examined a 50% and 300% increase in TM stiffness, represented by $n_\xi = 1.5$ and 4.0, respectively (Fig. 9). The normal physiological TM stiffness ($n_\xi = 1.0$) was defined to reproduce published histological measurements of SC dimensional changes as a function of IOP (Allingham et al., 1996; Van Buskirk, 1982).

With increasing TM stiffness, the TM deforms less in response to pressure gradients, which would reduce both TM stretch and SC shear stress, but to varying degrees (Fig. 9C). A 50% increase in TM stiffness was sufficient to eliminate virtually all shear stress experienced by SC cells, reducing the time-averaged and pulsatile amplitude of SC shear stress by 90% for the normal physiological value of r_{je} (Fig. 9B). Only a slightly larger decrease in time-averaged and pulsatile shear stress (96%) was reported for further increases in TM stiffness (up to 300%), indicating that SC shear stress is sensitive to relatively small (<50%) changes in TM stiffness, particularly under pulsatile conditions. Increasing TM stiffness also entirely eliminated the dependence of SC shear stress on r_{je}, markedly suppressing the elevated time-average and oscillatory shear stress that would otherwise occur under hypertensive conditions. In contrast, the effects of TM stiffness on TM stretch were less pronounced, with a 33% and 75% reduction in the time-averaged stretch for $n_\xi = 1.5$ and 4.0, respectively. Unlike the case for SC shear stress, TM stretch continued to increase with r_{je} when TM stiffness was elevated, although with a reduced sensitivity (or slope). These numerical simulations of Sherwood et al. (2019) demonstrate how increased TM stiffness may impair IOP mechanosensation by suppressing the magnitude of stretch or shear stress experienced by TM or SC cells in response to perturbations in outflow resistance.

Mechanosensation in the TM and SC is important because any debris within the anterior chamber, such as pigment or other cellular or extracellular material must eventually pass through the TM. There it may accumulate and potentially increase outflow resistance. Under conditions of normal TM stiffness, this perturbation may be detected based on changes in stretch acting on TM cells or shear stress acting on SC cells, and thereby induce a compensatory response to oppose the perturbation in outflow resistance. This compensatory response may include release of proteolytic MMPs to break down the resistive barrier (Bradley et al., 2001), VEGF to increase the permeability of the inner wall of SC (Reina-Torres et al., 2017) or NO to relax the TM, reduce outflow resistance and allow any accumulated material to pass more easily out of the TM. As the TM and SC becomes de-sensitized to perturbations in outflow resistance, debris and other matter that may accumulate in the TM and increase outflow resistance would remain undetected and thereby fail to elicit a compensatory response. Over time, this may lead to a progressive elevation in outflow resistance and IOP, ultimately resulting in ocular hypertension and potentially glaucoma. This may explain how increased TM stiffness could lead to increased outflow resistance, by inhibiting mechanosensation of IOP and de-sensitizing the TM and SC to perturbations in outflow resistance.

It is worth pointing out an additional mechanism by which TM stiffness, modulated by NO, may potentially amplify mechanosensation in the outflow pathway (Fig. 7B). NO relaxes the contractile elements in TM cells (Dismuke et al., 2014; Wiederholt et al., 1994), which will tend to reduce TM stiffness. For a given pressure drop across the outflow pathway, a reduction in TM stiffness will lead to increased TM stretch, which will further narrow SC

lumen, thereby increasing SC shear stress as well. In this manner, shear-induced NO production from SC may act in a feed-forward manner to amplify TM stretch and SC shear stress effectively increasing the mechanosensitivity of the outflow pathway to perturbations in outflow resistance or IOP.

6.11.1. The time scale of NO signaling in TM—A key property of NO signaling is that it is relatively fast, allowing it to be responsive on a time scale of IOP variation that occurs during the cardiac cycle. Shear mediated NO production occurs within seconds in vascular endothelia (Davies, 1995), which is important for controlling vasoregulation over short time scales. Shear-induced production of NO by SC cells is likely to be similarly fast, as is the effect of NO on TM cell contractility or SC permeability. This is because NO acts directly on sGC, VE-cadherin or other molecular targets to rapidly affect signal transduction or protein confirmation. Other mechanotransduction mechanisms, such as stretch-activated ion channels (Matthews et al., 2010) may be similarly fast. However, any mechanisms involving changes in gene expression will occur over a much longer time scale, requiring hours to days to exert their effect. This slower mechanotransduction response might utilize genes involved in NO signaling that are also associated with POAG, such as NOS3, CAV1/2, VEGF-C, ANGPT2, or TEK. Thus, the outflow pathway may exploit multiple mechanosensory mechanisms that operate over a range of different time scales. This would allow the outflow pathway to differentiate short versus long-term changes in the mechanical environment related to IOP.

To explore the rate at which shear-mediated NO production may affect outflow facility, we developed an oscillatory pressure system to reproduce the ocular pulse in cadaveric mice whilst measuring outflow facility. Our rationale was that the oscillatory pressure should impose oscillatory shear stress in SC (Sherwood et al., 2019), which we surmised would amplify the production of NO to lower outflow resistance. Within individual eyes, we measured outflow facility during alternating periods with and without modest oscillations in pressure (~1 mmHg pk-pk sinusoidal waveform at 10 Hz superimposed on an average IOP of ~8 mmHg). During periods when the oscillations were on, outflow facility was 16% greater than the facility measured in the same eye when oscillations were off (Fig. 10). When the pressure oscillations were applied (indicated by vertical blue bars in Fig. 10), there was an initial transient response that decayed within a few minutes, by which time the facility increase was apparent (Fig. 10). The facility increase was attributable to an active response of the eye to pressure oscillations because no change in facility was observed when repeating the experiment using a “mock eye” that consisted of a glass capillary and compliant tube that were chosen to match the physiological values of outflow resistance and ocular compliance for a real mouse eye.

To test the involvement of NO production, we performed the same experiment in the contralateral eye in the presence of 100 μM L-NAME, a pan-NOS inhibitor. L-NAME decreased the effect of pressure oscillations on outflow facility by nearly half, implicating NOS in the facility increase in response to pressure oscillations. Taken together, these studies reveal that pressure oscillations mimicking the ocular pulse lead to a near immediate increase in outflow facility with roughly half of this effect attributable to NOS. This is consistent with shear-induced NO production by SC in response to pulsatile shear stress,

which acts to immediately decrease outflow resistance. Such a fast-acting response to pulsatile pressure is consistent with proposed mechanosensory mechanism of shear-mediated NO signaling in the TM.

In the vascular system, there is evidence that oscillatory versus static shear stress will have a differential response on eNOS activation, expression, and NO production. In one study, oscillatory shear stress attenuated NO production compared to static shear stress in HUVEC (Yee et al., 2008). These data were supported by Ziegler et al. (1998), who demonstrated that oscillatory shear stress induced higher ET-1 mRNA expression but lower eNOS mRNA expression compared with static shear stress in bovine aortic endothelial cells. However, contradictory evidence in peripheral vasculature found that oscillatory shear stress enhances NO production (Li et al., 2005; Nakano et al., 2000). Future studies need to examine the effect of oscillatory shear stress on NO production in SC cells. Oscillatory flow is observable *in vivo* in both normal and glaucomatous eyes, however oscillatory flow is reduced in the SC lumen of glaucomatous eyes (Johnstone et al., 2011; Kerr et al., 2003), outlining the importance of oscillatory flow dynamics in glaucoma pathogenesis.

6.11.2. Mechanical model of shear-regulated NO production in the conventional outflow pathway—

SC is a collapsible endothelial-lined vessel. With increasing IOP, the TM expands outwards and the lumen of SC narrows (Grierson and Lee, 1974; Johnstone and Grant, 1973; Moses, 1977; Van Buskirk, 1982). This leads to at least two IOP-dependent biomechanical cues: stretch experienced by TM and SC cells and shear stress experienced by SC cells. We and others (Braakman et al., 2014; Bradley et al., 2001; McDonnell et al., 2020; Sherwood et al., 2019) have hypothesized that these biomechanical cues provide a feedback mechanism for IOP homeostasis. According to these models, IOP-induced stretch or shear stress induce TM or SC cells to respond in a manner that lowers IOP by stimulating pathways that decrease outflow resistance. Given the evidence described previously, eNOS and NO appear to be key players of this mechanism that would control relaxation of the TM and SC permeability to accommodate outflow resistance in order to regulate IOP (Fig. 7). Therefore, TM and SC cells should contribute directly to outflow resistance regulation. This notion contradicts the dogma that cells in the outflow pathway act as a passive filter, as Bárány (1954) and VanBuskirk and Grant (1974) postulated that outflow resistance was insensitive to metabolic inhibitors and changes in temperature respectively. Nevertheless, a recent study by Reina-Torres et al. (2020) reports a reduction in outflow facility when both glycolysis and oxidative phosphorylation are inhibited simultaneously, and that facility is heavily affected by temperature. This supports the idea that the cells in the outflow pathway actively regulate outflow. Thus, outflow tissues appear to have all the mechanisms necessary for regulate outflow in response to mechanical stimulus to maintain IOP homeostasis with NO as a mediator.

7. NO donating therapeutics

Given the hypothesized importance of NO in this IOP-sensitive feedback loop and that NO donors efficaciously lower IOP, targeting the NO pathway appears to be ideal for drug development to treat glaucoma. For example, in preclinical experiments topical or intravitreal administration of a number of different NO-donor molecules dramatically, but

transiently decrease IOP in rabbits (Behar-Cohen et al., 1996; Carreiro et al., 2009; Kotikoski et al., 2002). Nipradilol, a beta-blocker with a nitroxy group, demonstrates significant enhancement of IOP lowering in rabbits, compared to beta-blocker activity alone (Orihashi et al., 2005; Sugiyama et al., 2001). IOP lowering effectiveness of NO-donating molecules also translated to humans. Organic nitrates given intravenously (nitroglycerin) or orally (isosorbide diniatrate), dose-dependently lower IOP in normal or glaucomatous human eyes that did not correlate with hemodynamic changes (Wisznia et al., 1970; Wizemann and Wizemann, 1980). Moreover, patients infused with L-arginine, the endogenous substrate for NO generation also displayed a transient IOP-lowering (Chuman et al., 2000).

A recent strategy has been to add NO-donating moieties to drugs already approved to treat glaucoma. For example, a series of NO donating modifications were made to the carbonic anhydrase inhibitor, dorzolamide. The best candidate, unfortunately only showed modest increase (1 mmHg) in IOP lowering over equimolar dorzolamide in rabbits, and thus was not pursued (Steele et al., 2009). In parallel, three different analogues of latanoprost were produced and examined preclinically. The first was NCX139, having a latanoprost core and molsidomine as its NO donor (Impagnatiello et al., 2011). NCX139 demonstrated significant IOP-lowering activity in ocular hypertensive rabbits as well as in normotensive and glaucomatous dogs; however, it did not appear significantly more efficacious than latanoprost alone. The second, NCX125, was formed by combining latanoprost with glycerol 1, 3 dinitrite as the NO donor (Borghi et al., 2010). In rabbit, dog, and nonhuman primate models NCX125 effectively lowers IOP better than latanoprost alone. The third, latanoprostene bunod (LBN, AKA: BOL-303259-X) was produced by combining latanoprost with butanediol mononitrite as the NO donor (Cavet and DeCory, 2018). LBN was found to be more efficacious than latanoprost at lowering IOP in three different animal models (rabbit, dog, and non-human primate) (Krauss et al., 2011). IOP lowering was maximal at 1–2 h post treatment and the largest effects were observed in ocular hypertensive rabbits, who traditionally respond poorly to PGAs. Moreover, LBN lowers IOP by 1.23 mmHg in FP receptor knockout mice (Cavet and DeCory, 2018). Of the three modified versions of latanoprost, LBN was advanced to human studies.

A series of 6 clinical trials were executed, testing the safety and efficacy of LBN. The phase I study, KRONUS demonstrated the efficacy of LBN over a 24 h period, and safety over 14 days in a small number of Japanese subjects (n = 24) (Araie et al., 2015). Next, the phase II study, VOYAGER, was a dose-ranging study that examined effects of LBN compared to latanoprost over 28 days (n = 413 subjects with open angle glaucoma or ocular hypertension). Maximum effects were observed at 0.024% LBN, demonstrating 1.2 mmHg IOP lowering advantage over latanoprost (Weinreb et al., 2015). The next phase II trial was CONSTELATION, looking at the 24 h IOP effects of LBN in 24 patients with ocular hypertension or early POAG. Like latanoprost, LBN demonstrated superiority to timolol during nocturnal hours. A pair of phase III nearly identical studies APOLLO (Weinreb et al., 2016) and LUNAR (Medeiros et al., 2016) were conducted, examining the safety and efficacy studies of LBN 0.024% in patients with open-angle glaucoma or ocular hypertension. In each study, LBN was compared to timolol over a 3-month period. Pooled data from the studies showed that LBN was non-inferior to timolol at every time point

(Weinreb et al., 2018). Moreover, pooled results from the safety extension phases of both studies demonstrated that the IOP reduction by LBN was maintained up to 12 months (Weinreb et al., 2018). The sixth human trial tested efficacy and safety of LBN in a Japanese cohort of patients with ocular hypertension or glaucoma for one year. Here, a mean reduction in IOP of 22% was achieved. In all studies, LBN was well tolerated, with a safety profile not significantly different from latanoprost.

Another prostaglandin analogue, bimatoprost was also modified for dual action capabilities by esterification with the NO-donating moiety 6-(nitrooxy) hexanoic acid (NCX 470). In preclinical studies, NCX 470 effectively lowers IOP more than equimolar bimatoprost in three animal models of glaucoma (rabbit, monkeys and dogs) (Impagnatiello et al., 2015). Subsequently, NCX 470 was advanced to human clinical trials. Here, a recently completed phase II clinical study evaluated the safety and efficacy of topical NCX 470 in lowering IOP in 656 patients with ocular hypertension or open-angle glaucoma. Three different concentrations of NCX 470 (0.021%, 0.042%, and 0.065%) were compared to latanoprost (clinicaltrials.gov identifier NCT03657797NCT03657797). While not yet published, Nicox announced positive results from this phase II trial and as a result secured funding to support a phase III trial (www.nicox.com).

Unfortunately, poor corneal penetration, short duration of action, and a narrow therapeutic index of NO-donors, so far have limited their clinical utility in treating glaucoma. In response, recent reports show that others are developing novel agents, such as NO-releasing polydiazeniumdiolate, which *in vitro* releases NO steadily over 48 h, however efficacy and long-term effects on TM cell contractility and IOP in living rabbits appears minimal (Jeong et al., 2020). Using another strategy to address corneal penetration, microporous silica nanoparticles were loaded with SNP, and a single topical drop in mice demonstrated significant IOP lowering over 30 h (vs. 30 min for SNP alone) (Hu et al., 2018). A third platform involved a two-step process to ensure that NO was released at its site of action for IOP lowering, the conventional outflow pathway. The goal was *in situ* NO release via enzyme biocatalysis in the JCT to increase outflow facility. This was tested in two phases: the first was intracameral injection of encapsulated β-galactosidase, enabling embedding in the JCT. The second controlled release of NO in TM by perfusing β-gal-NONOate-loaded liposomes into eyes and observing increased outflow facility (Chandrawati et al., 2017).

While steady progress has been made towards developing a viable glaucoma therapy involving NO delivery to conventional outflow tissues, two large hurdles remain. Most prominent is access of NO to cells of the JCT and sustained delivery of NO to modify flow passageways continually. Perhaps NO-donating drugs can be developed and married with a suitable biodegradable implants such as was done with bimatoprost and the Novadur™ platform, which recently received the U.S. food and drug administration approval (Craven et al., 2020). Ocular drug development for glaucoma is moving in this direction due to ocular surface problems, poor penetration and patient adherence issues with topical forms of medications.

8. Conclusions and future directions

In summary, NO plays a major role in regulation of IOP and outflow resistance, mediating physiological responses in the TM, SC, and distal vasculature. Shear stress is the main mechanical stimulus regulating NO production in the outflow pathway, and several factors influence how shear stress is detected or transduced such as TM stiffness, oscillations, and acute verses chronic shear stress. There is still a gap in knowledge as to how the SC cells sense and respond to mechanical stimuli such as shear stress, and how these signals regulate NO dynamics. Mechanisms for shear stress-induced NO production are well known in blood vasculature, and blood vascular endothelial cells share many phenotypic characteristics with endothelial cells of the SC (Kizhatil et al., 2014). As such, researchers have identified several mechanosensory pathways known in blood vascular endothelial cells that are promising candidates for mechanosensation and NO regulation in SC and other tissues of the outflow pathway. Here, we have summarized some of the main candidates including CAV1 and caveolae, PIEZO1, the VEGFR/VE-cadherin/PECAM-1 complex, the Tie2/PI(3)K/Akt signaling pathway, and the glycocalyx. However, SC also exhibits several unique physiological characteristics; fluid flow and pressure is basal to apical unlike blood vasculature (Ethier et al., 2004), and the SC is a hybrid blood/lymphatic vessel as it has to maintain blood-aqueous barrier and regulate fluid to flow out of eye (Kizhatil et al., 2014). Thus, it is important to apply our understanding of the systemic vasculature to the unique nature of the SC when investigating these mechanosensory pathways and the role they play in the outflow pathway. This new knowledge will hopefully lead to enhanced understanding of outflow dynamics and glaucoma pathogenesis and provide more therapeutic targets for the treatment of POAG.

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Abbreviations:

C	outflow facility
CaM	calmodulin
CAV1	caveolin-1
CM	ciliary muscle
eNOS	endothelial nitric oxide synthase
Hsp90	heat shock protein 90
KO	knock out
IOP	intraocular pressure
JCT	juxtacanicular tissue

NO	nitric oxide
POAG	primary open angle glaucoma
SC	Schlemm's canal
sGC	soluble guanylyl cyclase
TM	Trabecular Meshwork
VEGF	vascular endothelial growth factor
EDRF	endothelium-derived relaxing factor
NOS	NO synthase
nNOS	neuronal NOS
iNOS	inducible NOS
eNOS	endothelial NOS
FAD	Flavin adenine dinucleotide
FMN	flavin mononucleotide
GTP	guanosine 5′-triphosphate
PIEZO1	Piezo-type mechanosensitive ion channel component 1
VE	cadherin, vascular endothelial cadherin
PECAM-1	platelet endothelial cell adhesion molecule-1
cGMP	guanosine 3′,5′-monophosphate
ET1	endothelin-1
MLCK	myosin light chain (MLC) kinase
VSM	vascular smooth muscle
L-NAME	L-NG-nitroarginine methyl ester
ATP	adenosine triphosphate
AMP	adenosine monophosphate
ADMA	asymmetric dimethylarginine
EVP	episcleral venous pressure
MMPs	matrix metalloproteases
LBN	atanoprostene bunod

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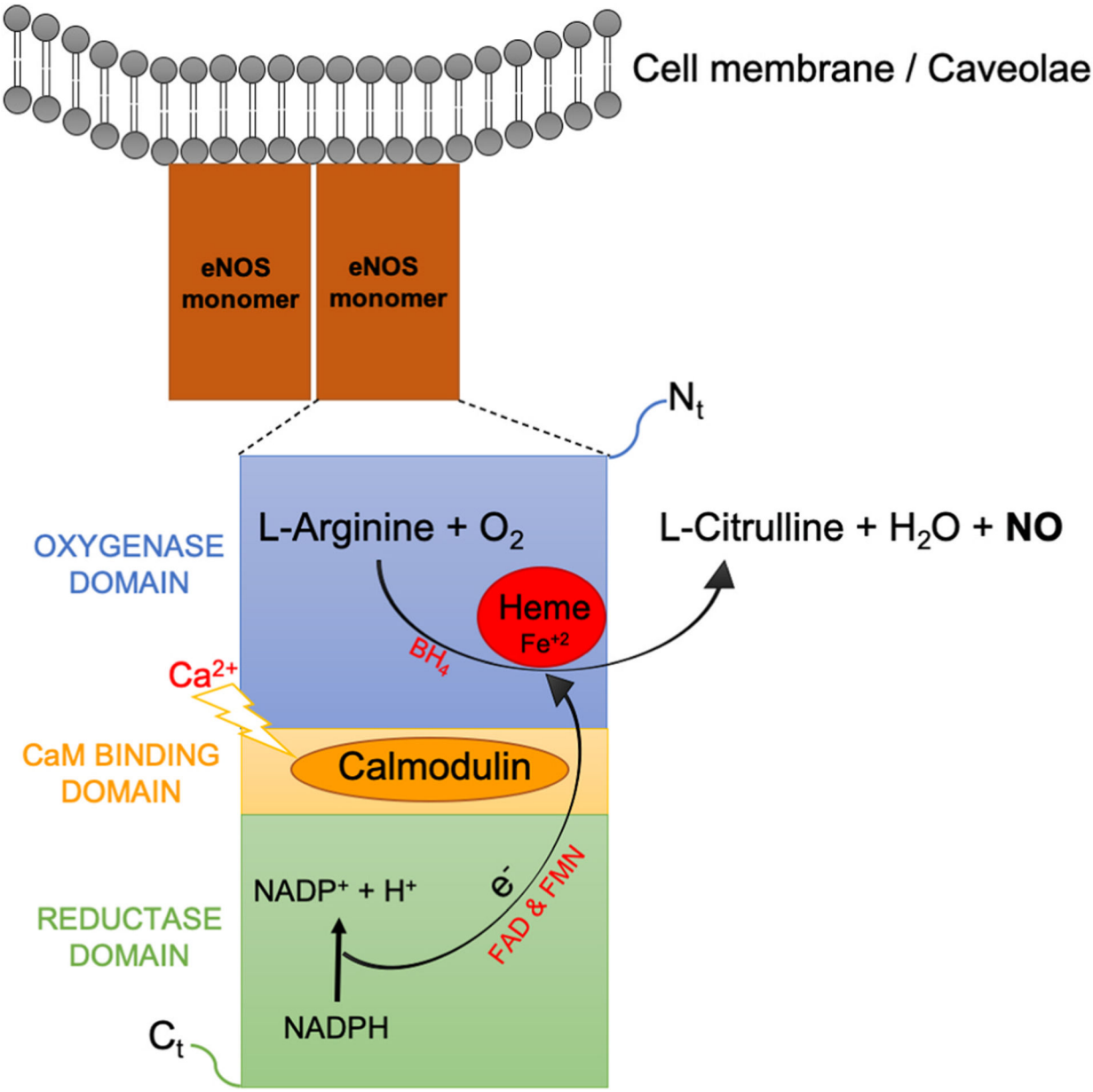

Fig. 1.

Schematic representation of eNOS structure and chemical interactions for NO production. For efficacious NO production eNOS homodimers localize at the cell membrane, preferably at caveolae. Within each monomer, electrons (e^-) released form the conversion of NADPH to $NADP^+$ and H^+ in the reductase domain are transported to the oxygenase domain. Transport is facilitated by the cofactors FAD and FMN and the presence of Ca^{2+} dependent Calmodulin (CaM) at the CaM binding domain. In the oxygenase domain, the presence of a Heme moiety and the cofactor BH_4 catalyze the oxidation of L-Arginine to L-Citrulline, H_2O and NO.

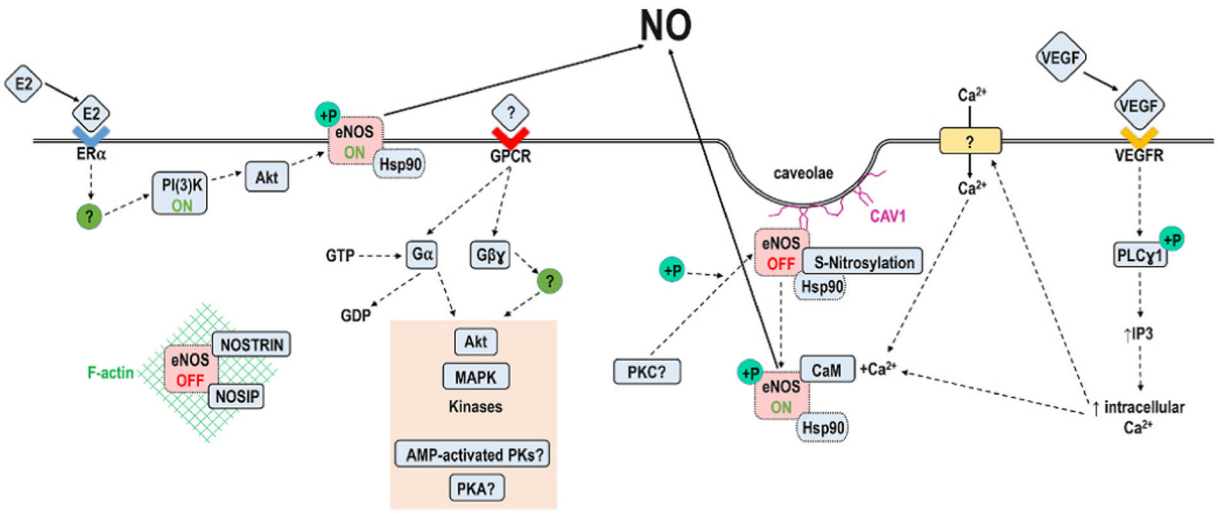

Fig. 2.

Modulators of endothelial nitric oxide synthase (eNOS) activity. eNOS activation requires the interplay of a variety of cell surface receptors and channels, scaffolding proteins, cytoskeletal elements, signaling molecules, and protein modifications. Shown are the elements, relationships and signaling that are proposed to be involved in eNOS activation/inactivation in Schlemm's canal endothelia. Hsp90 promotes the affinity of eNOS for CaM, and it is necessary for Akt to interact with eNOS. eNOS is negatively regulated by interaction with Cav1. NO further negatively regulates Hsp90 and subsequently eNOS activity via S-nitrosylation. Estrogen exhibits rapid, nongenomic activation of the PI(3)K/Akt/eNOS pathway, possibly via ERα. VEGF stimulates phosphorylation of PLCγ1, increasing levels of IP3 and subsequently mobilizing intracellular calcium necessary for CaM-induced eNOS activation and for the opening of calcium Ca^{2+} channels. NOSTRIN and NOSIP regulate eNOS internalization and trafficking and both inhibit eNOS function. Other biochemical signals that can activate eNOS are mostly mediated through GPCRs. Downstream of GPCR activation, other proteins potentially involved in the regulation of eNOS activity include PKA, PKC, MAPK, and AMP-activated protein kinases. GPCR: G-protein coupled receptor. NO: nitric oxide, Akt/PKB: v-akt murine thymoma viral oncogene homolog; TK: thymidine kinase; MAPK: mitogen-activated protein kinase; AMP-activ PK: AMP activated protein kinase; PKA: protein kinase A; PKC: protein kinase C; HSP90: heat-shock protein 90; CaM: calmodulin; Ca^{2+}: calcium, E2: estrogen, ER: estrogen receptor, VEGF: vascular endothelial growth factor, PLC: phospholipase C, GTP: guanosine triphosphate; IP3: Inositol trisphosphate; NOSIP: eNOS interacting protein; NOSTRIN: eNOS trafficking inducer protein; F-actin: filamentous actin.

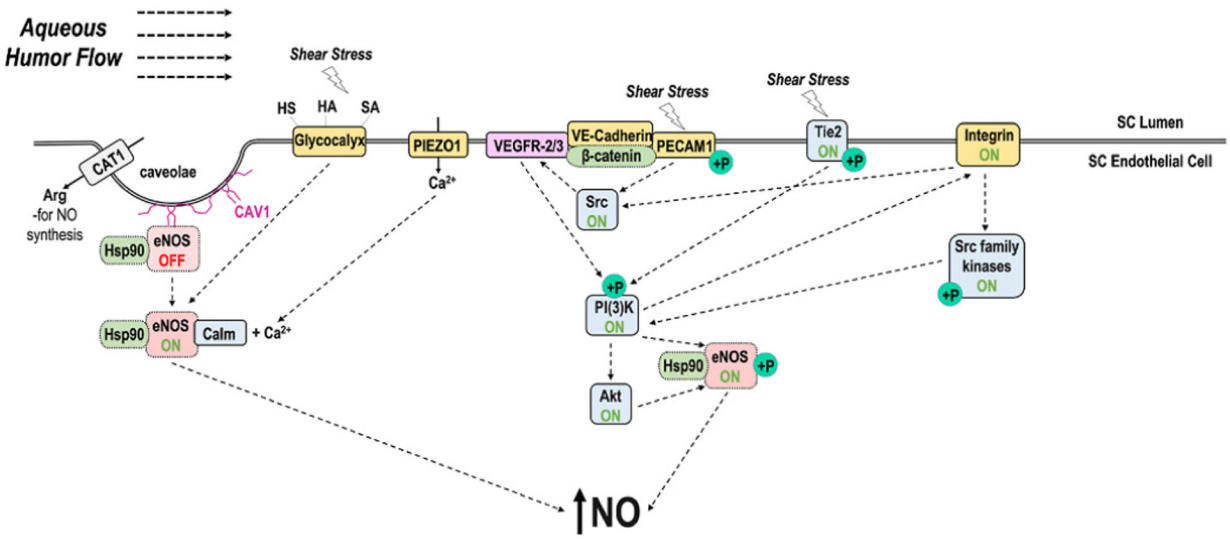

Fig. 3.

Schematic summarizing proposed signaling pathways involved in shear stress-induced regulation of nitric oxide (NO) production by Schlemm's canal (SC) endothelial cells. Shear stress induces a variety of physiological responses in endothelial cells, with some pathways inducing eNOS activation and NO production. Caveolae and caveolin 1 (CAV1) act as mechanotransducers in SC endothelial cells, initiating and integrating signaling cascades in response to shear stress. CAV1 binds to and negatively regulates endothelial nitric oxide synthase (eNOS), and Hsp90 is necessary for Ca^{2+}-dependent calmodulin-induced eNOS dissociation from CAV1 and for Akt-induced phosphorylation of eNOS. CAT1 and several molecules that regulate intracellular Ca^{2+} concentration localize in caveolae, enabling efficient and rapid eNOS activation in response to shear stress. The VEGFR/VE-cadherin/PECAM-1 mechanosensory complex regulates eNOS activity and NO production, and VEGFR-2 associates with CAV1 and is localized in endothelial caveolae. PECAM-1 is required for the mechanosensation of shear stress and Src activation, while VE-cadherin enables signal transmission to PI(3)K, which goes on to activate integrins and mediate other signaling pathways (such as Akt and eNOS). The glycocalyx also mediates mechanosensation of shear stress and subsequent downstream eNOS signaling. The Tie2/PI(3)K/Akt signaling pathway regulates shear stress-induced eNOS activity and NO. Finally, PIEZO1 is activated by shear stress, potentially regulating shear stress-induced elevation of intracellular Ca^{2+} and NO production.

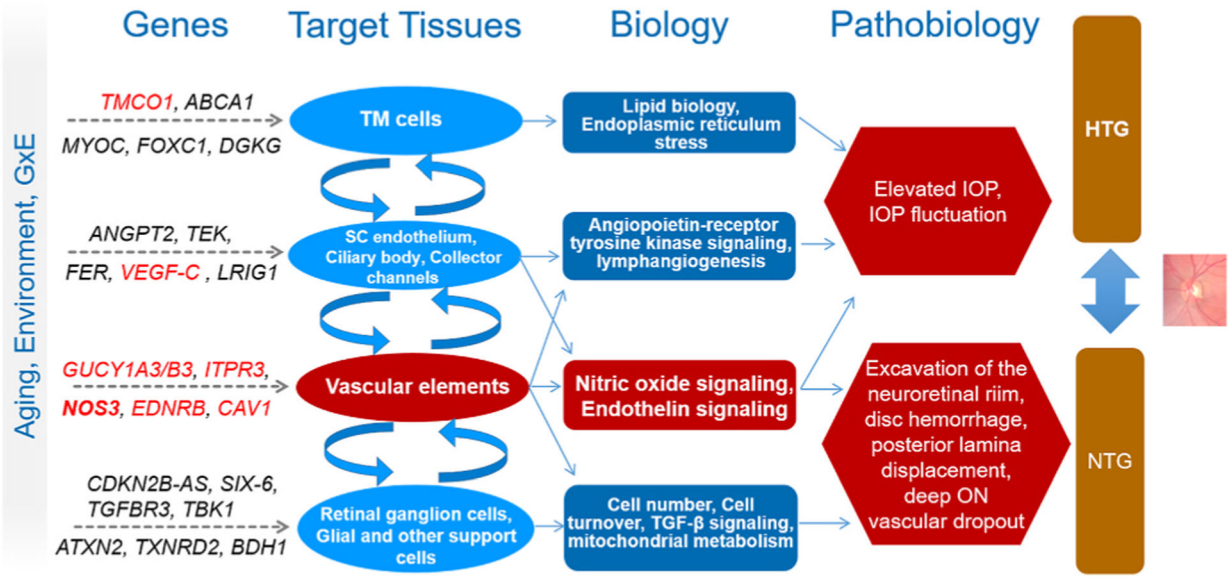

Fig. 4.

A schematic showing multifactorial pathobiology of primary open angle glaucoma (POAG), involving complex genetics, environment and target tissues. POAG is a hereditable disease characterized by optic nerve damage, with the primary risk factor and currently viable treatment target being intraocular pressure (IOP). Several genes are associated with dysfunctions leading to POAG affecting both regulation of IOP and the optic nerve health. Genes highlighted in red may influence NO signaling. GxE: gene environment interactions; HTG: high tension glaucoma; NTG: normal tension glaucoma.

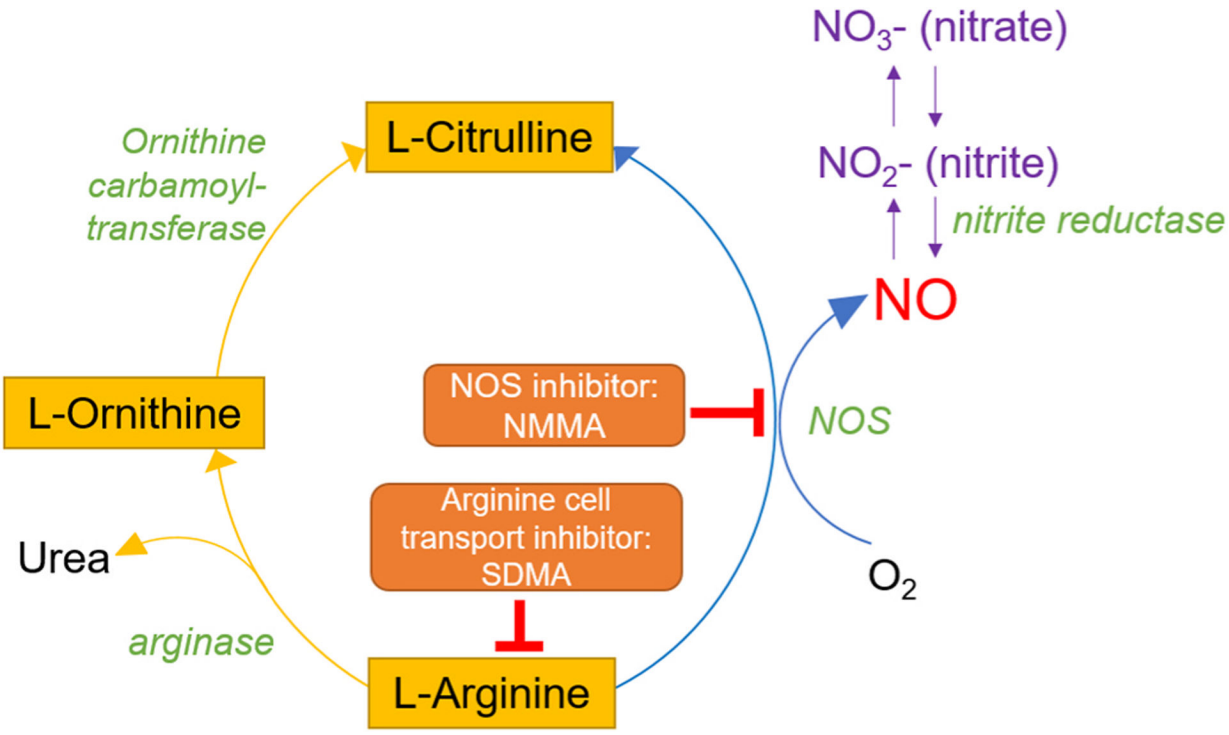

Fig. 5.

The Nitrate – Nitrite – Nitric oxide signaling pathway. Oxidation of L-Arginine results in production of nitric oxide (NO) and L-Citrulline. NO, in turn, can react with oxygen to form nitrites and nitrates. NO can be liberated from nitrites and nitrates through reduction catalyzed by nitrite reductase. Arginine is also a substrate for arginase in the urea cycle, producing a urea and L-ornithine, which in turn can also be converted into L-Citrulline. These reactions can be altered by drugs such as NOS inhibitors and symmetric dimethylarginine (SDMA).

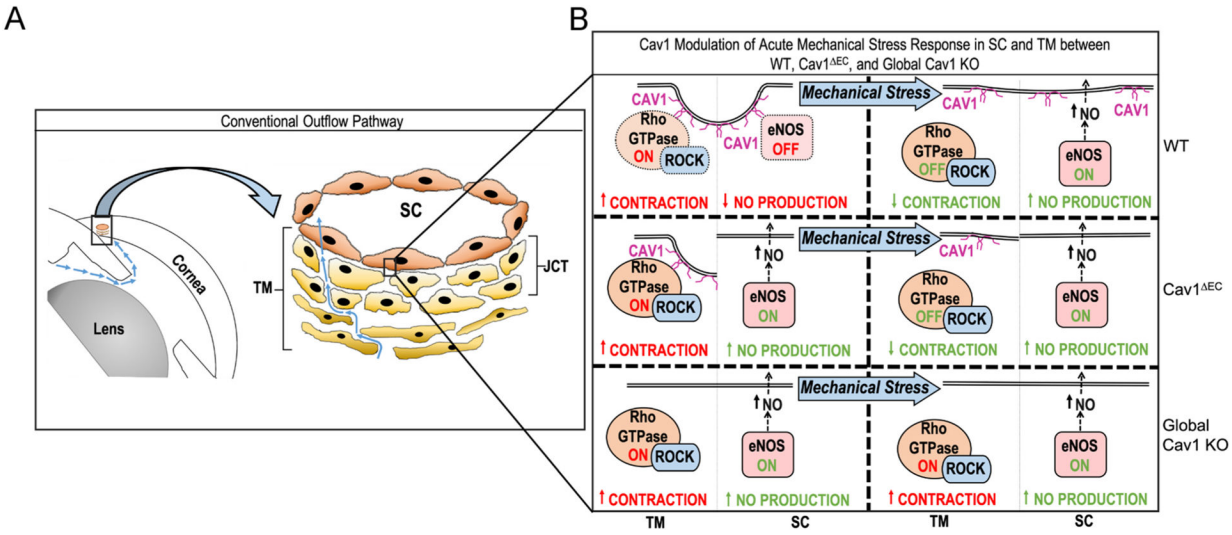

Fig. 6.

Schematic illustrating the differential role of caveoli in the physiological response of TM and SC cells to mechanical stress. (A) Illustration of the conventional outflow pathway. (Left) Low magnification showing lens, cornea, iridocorneal angle and direction of aqueous humor flow (blue arrows). (Right) Higher magnification depicting Schlemm's canal (SC), trabecular meshwork (TM) and juxtacanicular tissue (JCT) with a preferential pathway for aqueous humor flow (blue arrows). (B) Flow diagram demonstrating the proposed status of Rho GTPase (in TM) and eNOS (in SC) before and after mechanical stress in three different types of mice: WT, endothelial CAV1 KO ($Cav1^{\Delta EC}$), and global CAV1 KO, and the resultant effect on nitric oxide (NO) production or cellular contraction.

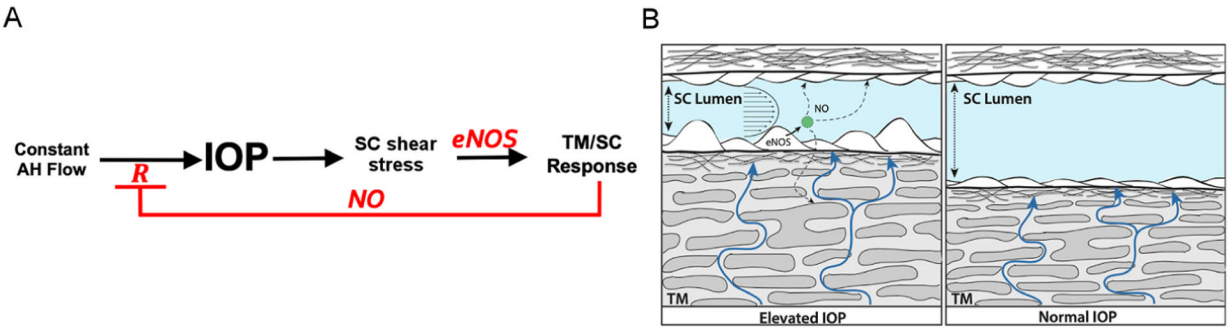

Fig. 7.

A model of shear stress-mediated mechanosensation of intraocular pressure (IOP). A) Diagram of homeostatic feedback loop where outflow resistance (R) determines IOP based on the magnitude of aqueous humor flow, which is typically constant for a living eye. IOP in turn defines the magnitude of shear stress experienced by Schlemm's canal (SC). This shear stress may elicit a mechanobiological response by trabecular meshwork (TM) or SC cells, specifically the release of nitric oxide (NO). NO, in turn, feeds back to reduce R and thereby return IOP towards a homeostatic set-point. B) A schematic of shear-induced NO production by SC cells at elevated IOP (left panel) relative to normotensive conditions (right panel). NO (green) may diffuse upstream to reach the TM or may act elsewhere on SC cells or on collector channels. Illustrations provided by Jason Y. H. Chang, PhD.

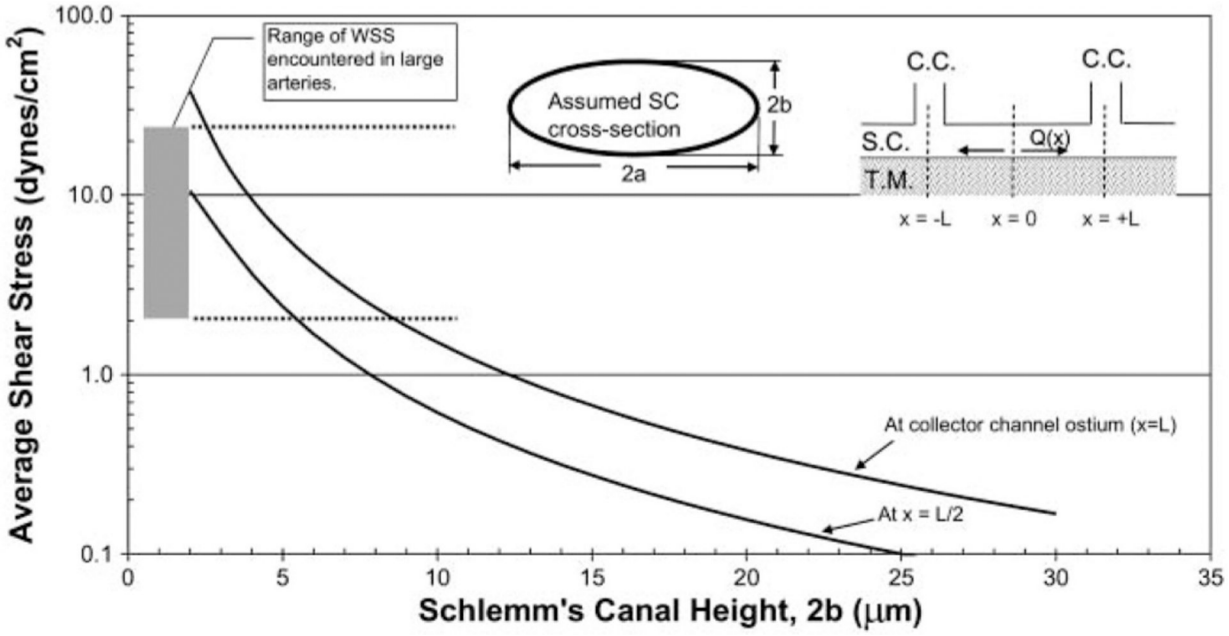

Fig. 8.

Mathematical predictions of the shear stress acting on Schlemm's canal (SC) cells in a human eye. SC lumen is modelled as elliptical in cross-section with semi-major and semi-minor axes *a* and *b*, respectively. Decreasing *b*, corresponding to a narrowing of SC that occurs with elevated IOP, increases the shear stress acting on SC cells. The shear stress is larger near collector channel ostia, where it may approach levels experienced by vascular endothelia in large arteries. CC: collector channels; WSS: wall shear stress; L: distance between CC; x: given position in SC. Reproduced with permission from Ethier et al. (2004).

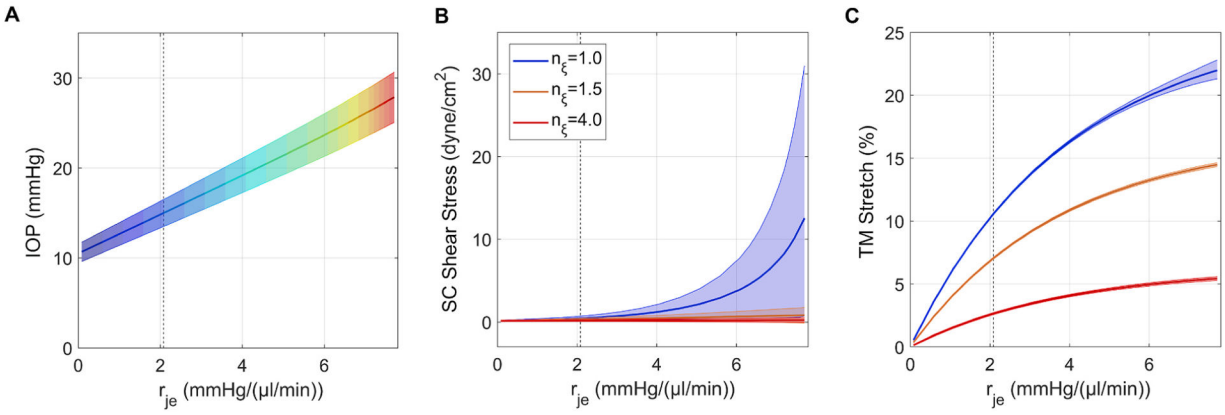

Fig. 9.

Mathematical predictions of the effects of the ocular pulse on the oscillatory intraocular pressure (IOP), (A), shear stress in Schlemm's Canal (SC); (B) and stretch in the trabecular meshwork (TM) (C) as a function of outflow resistance generated by the inner wall of SC and juxtacanicular tissue (r_{je}). Shaded regions represent the pulsatile amplitude, while the central curve represents the time averaged value over one cardiac cycle. Different curves in panel B and C represent different values of TM stiffness, including physiological TM stiffness (n_ξ = 1.0) and 50% and 300% increases in TM stiffness (n_ξ = 1.5 and 4.0, respectively). SC shear stress is evaluated at the collector channel ostium. Reproduced from Sherwood et al. (2019) under Creative Commons Attribution License of the Royal Society.

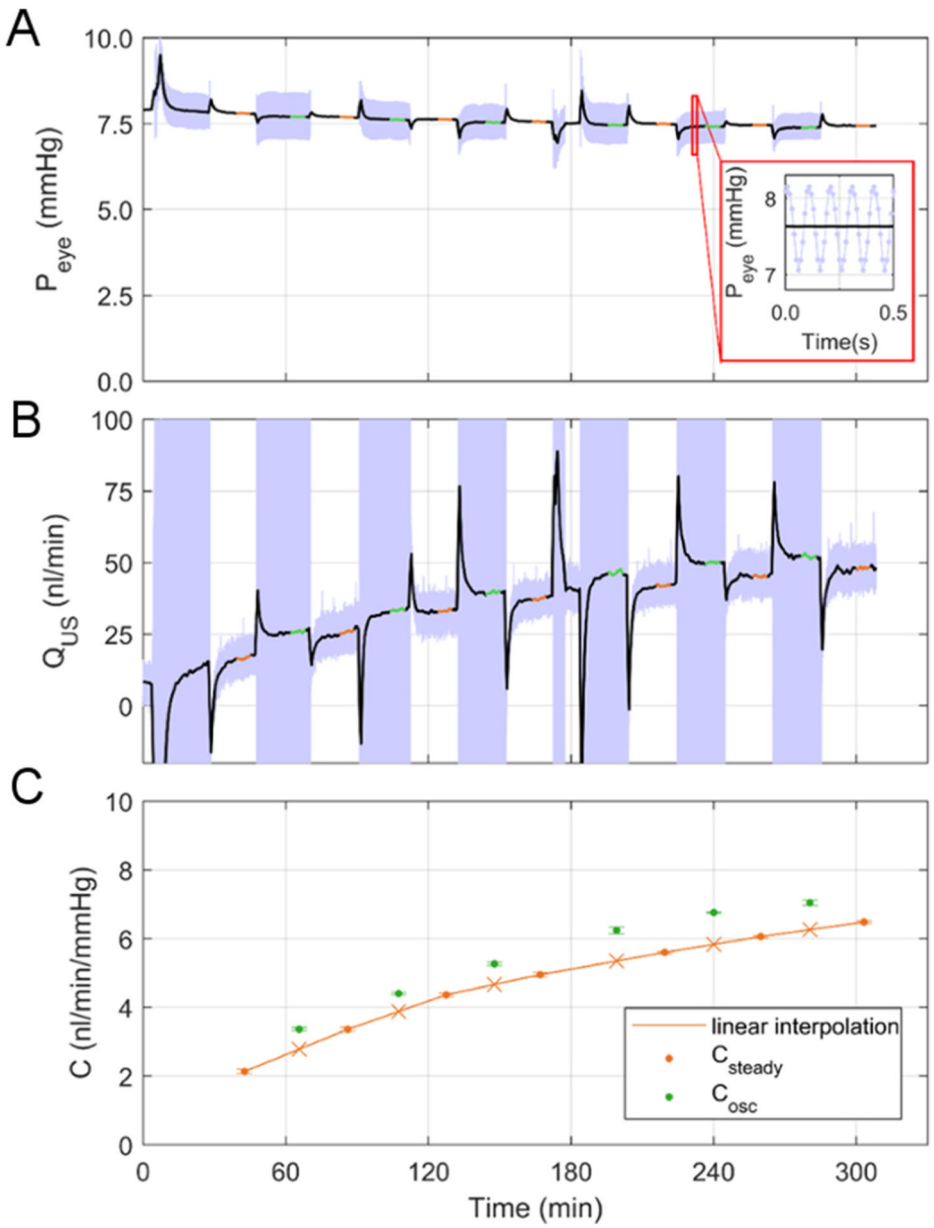

Fig. 10.

Effect of pressure pulsations that mimic the ocular pulse on outflow facility in cadaveric mouse eyes. A) IOP is maintained at a baseline pressure of 7.5–8.0 mmHg with alternating periods with or without sinusoidal pressure pulsations (1.0 mmHg pk-pk at 10 Hz). Pressure pulsations are indicated by blue shading. The slight decay in baseline pressure is due to a change in the outflow resistance of the eye whilst the flow sensor between the eye and pressure reservoir has a constant resistance (~10 mmHg/(ul/min)). B) The mean flow rate entering the eye, with the time-averaged value shown in black and oscillations shown in blue. C) The measured value of outflow facility under steady (orange circles) and pulsatile (green circles) conditions and a linear interpolation predicted of steady condition outflow facility during pulsatile periods (orange crosses). Outflow facility under oscillatory

conditions is elevated with respect to that under steady conditions. The upward trend in outflow facility over time is likely due to anterior chamber deepening.

Table 1

Compilation of studies assessing the effect of NO stimulation or inhibition in IOP and/or outflow facility (C).

Species	Treatment	IOP	C	Reference
Mice	transgenic mice overexpressing eNOS	1.8 mmHg reduction compared to WT	>2-fold increase compared to WT	Stamer et al. (2011)
	100 μM L-NAME in transgenic mice overexpressing eNOS		C reduction, compared to WT	
Mice	100 μM SNAP – WT mice (IC) 100 μM SNAP – eNOS-GFPtg mice (IC) 10 μM L-NAME – WT mice (IC) 50 μM cavtratin – WT mice (IC) 50 μM cavtratin – eNOS-GFPtg mice (IC)		62 ± 28% increase No effect 36 ± 13% decrease 19 ± 12% decrease 39 ± 25% decrease	Chang et al. (2015)
Mice	eNOS KO 160 μg SNP or SNAP in WT (T)	4.3 mmHg increase compared to WT SNP: 5 mmHg reduction SNAP: no effect	29% reduction compared to WT	Lei et al. (2015)
	1 mM SNP or 100 μM SNAP – perfused in WT		SNP: 2.5-fold increase SNAP: 2.9-fold increase	
Mice	10 μM L-NAME (IC)		Non-significant reduction	Elliott et al. (2016)
Mice	100 μM L-NAME (IC) eNOS KO		28.3% reduction 35.8% reduction compared to WT	Kizhatil et al. (2016)
Mice	β-gal-NONOate-loaded liposomes (IC)		84% [23, 177%] increase	Chandrawati et al. (2017)
Mice	Breathing NO for 40 min - WT	3.5 mmHg reduction	13.7% ± 14.6% increase	Muenster et al. (2017)
Rabbit	0.03–0.1 g % (for IOP) and 0.1% (for C) Nitroglycerine (T)	Dose dependent reduction, up to 30% in 1–2 h	42 ± 15% in 1 h	Nathanson (1992)
Rabbit	20 mM SIN-1 (IV) 20 mM SIN-1 (IC) 20 mM SNAP (IV)	mean reduction of 71% ± 5% in 1 h decrease of 68% ± 8% in 1 h reduction of 72% in 1.5 h		Behar-Cohen et al. (1996)
Rabbit	intravenous drip-infusion of 10 ml of L-arginine (1 gr)	Reduction of 4 mmHg		Chuman et al. (2000)
Rabbit	5 mg/kg L-NAME (V)	24 ± 2% reduction		Kiel et al. (2001)
Rabbit	nitrosocaptopril 12.3 mg (IC) captopril 10.9 mg (IC) SNP 13.1 mg (IC) 8-BrcGMP 22.3 mg (IC)		Increase of C_1 = 80% and C_2 = 74% Increase of C_1 = 69% and C_2 = 64% Increase of C_1 = 35% and C_2 = 33% Increase of C_1 = 12% and C_2 = 2%	Kotikoski et al. (2003)
Rabbit	15 μl 1% (w/v) L-NAME (T)	Normotensive: No effect Hypertensive: Max 12 mmHg reduction at 1 h		Giuffrida et al. (2003)
Rabbit	1% w/v SNP (T)	2 mmHg reduction in 1 h		Carreiro et al. (2009)
Lamb	single dose of NO (80, 250, 500, or 1000 ppm) for 60 min for 4 days (T)	reduction in a dose-dependent manner (0.43 mmHg/ppm NO)		Muenster et al. (2017)
Porcine	(DETA)-NO 100 μM		Increase of 0.86 ± 0.10 μl/min/ mmHg− 1	Dismuke et al. (2008)

Species	Treatment	IOP	C	Reference
Porcine	(DETA)-NO 100 μM		220% increase	Ellis et al. (2009)
Monkey	Nitroglycerin (IOP: T, 0.1%) (C: IC, 1 mM) Hidralazine (IOP: T, 0.1%) (C: IC, 10 μM)	Decrease of 4.4 ± 2.1 mmHg Decrease of 3.2 ± 0.9 mmHg	Increase of 92% Increase of 28%	Schuman et al. (1994)
Monkey	SNP (IOP: 500 μg, 4× T) (C: 1 mM, IC) L-NAME (IOP: 0.5 mg, 2× T)	reduction of 20% at 3 h No significant effect	increased by 77%	Heyne et al. (2013)
Human	V drip infusion of 100 ml of L-arginine (10.0 g) solution	2 mmHg reduction after 10 min		Chuman et al. (2000)
Human	1 μM, L-NAME 10 mM, L-NAME 20 mM L-arginine 10 mM L-NAME 10 mM sodium nitroprusside		3.8 ± 1.8% reduction 10.4 ± 2.2% reduction 1.9 ± 1.6% reduction 1.0 ± 1.9% reduction 10.2 ± 2.2% increase	Schneemann et al. (2002)

Abbreviations: WT = wild type; L-NAME = L-N^G-nitroarginine methyl ester; SNAP = S-Nitroso-N-acetylpenicillamine; GFPtg = green fluorescent protein transgenic; SNP = sodium nitroprusside; SIN-1 = 3-Morpholinosydnonimine IV = intravitreal, IC = intracameral, T = topical, V = intravenous, C = outflow facility (C_1 and C_2 refer to sequential C measurements).

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Table 2

Nitric oxide synthase isoform gene expression (average UMI) in different ocular cell clusters isolated from human outflow tissues. For each gene, saturated yellow represents highest expression among molecularly defined cell types, saturated blue represents lowest expression, and intermediate shades of color represent intermediate expression.

	SC - Lymphatic endothelium	Vascular endothelium	TM - Myofibroblast	TM - Fibroblast	CM - Smooth muscle cell
NOS3	0.4190	0.6015	0.0145	0.0062	0.0022
NOS2	0.0000	0.0000	0.0004	0.0005	0.0013
NOS1	0.0110	0.0399	0.0199	0.0119	0.0207

UMI: unique molecular identifiers, SC: Schlemm's canal, TM: trabecular meshwork. Adapted from Patel et al. (2020).

Table 3

NO values measured in aqueous humor of patients with different types of glaucoma.

cataracts	POAG	CCAG	ACAG	NVG	Units	Reference
35.5 ± 1.3	31.3 ± 2.5	42.2 ± 2.6	64.8 ± 7.6*	67.6 ± 8.2*	μmolar	Chang et al. (2000)
27.1 ± 3.6			83.2 ± 6.7*		μmolar	Chiou et al. (2001)
86.92 ± 11.23	72.72 ± 11.21*				μmolar	Doganay et al. (2002)
27.0 ± 2.9	36.2 ± 3.3*	47.6 ± 3.4*		65.8 ± 12.3*	μmolar	Tsai et al. (2002)
24.6 ± 10.2	25.7 ± 16.2				μmolar	Kotikoski et al. (2002)
259.20 ± 72.62	156.82 ± 54.78*				μmol/mg protein	Galassi et al. (2004)
56.6 ± 11.0					μmolar	Fard et al. (2010)
23.01 ± 12.49 (0.76)					ng/ml (μmolar)	Borazan et al. (2010)
34.1 ± 6.2	53.4 ± 11.2*	82.5 ± 11.3*			μmolar	Ghanem et al. (2011)

*Authors report statistical difference from cataracts. POAG: primary open angle glaucoma; CCAG: chronic closed angle glaucoma; ACAG: acute closed angle glaucoma; NVG: neovascular glaucoma.